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ON THE FUNCTION OF THE PHARYNGEAL RIDGES IN THE LARVAE OF *CALLIPHORA ERYTHROCEPHALA*

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(With 3 Figures in the Text)

"THE Diptera larvae can be divided from an ethnological point of view into four categories: and the members of each, though of diverse systematic affinities, possess in common certain structural characters, which Keilin (1912) considers to be adaptations to the manner of life. The categories are as follows: (i) Saprophagous larvae: those which feed on decayed vegetable matter or the excrement of herbivorous animals; these have a buccal armature similar to that of *Calliphora* or *Lucilia*: the ventral surface of the basilar piece is feebly chitinized and has a number of longitudinal ridges projecting into the lumen of the pharynx. (ii) Phytophagous larvae, which feed on living plants, as leaf-miners, root-miners, etc.: these have the bucco-pharyngeal armature more concentrated, the basilar piece more chitinized, with ridges feebly developed or absent, the mandibles modified as several toothed scrapers. (iii) Parasitic larvae: their buccal armature resembles that of parasitic Tachinidae. (iv) Parasitic larvae—carnivorous larvae: It is now known from the researches of Portchinsky (1910), and later of Keilin himself, that a good number of larvae found in excrement are not really coprophagous, but live on other larvae or other small animals found with them in the excrement. These carnivorous larvae attack their prey, perforating its skin, and sucking its contents. For instance, the larvae of *Graphomyia maculata*, found in liquefied decomposing vegetable matter, feed on larvae of *Eristalis* and pupa of Tipulidae, those of *Calliophris riparia*, found among moss, feed on small oligochaete worms and larvae and pupae of Psychodidae. These carnivorous larvae have certain characters in common. Thus the mandibles are sharp, piercing hooks and the basilar piece of the armature is very long, narrow and strongly chitinized and entirely without the internal longitudinal ridges." (Scott, 1915.)

If the ventral surface of the pharynx of larvae of *C. erythrocephala*, previously treated with caustic potash and then mounted on a slide, be examined under the low power of the microscope, these ridges can be easily recognized from outside. They run longitudinally along the whole length of the pharynx. But their exact character can only be seen in a transverse section. In *Calliphora* each one has the shape of a T, which arises from the

basilar piece of the buccal armature, the two horizontal limbs come to meet with the corresponding pieces of the opposite sides, thus an oval or a circular cavity being enclosed. These ridges, on account of their peculiar shape, have been styled by Holmgreen (1904) as T-shaped ridges, and they project well into the lumen of the pharynx. The pharyngeal cavity is thus divided into two parts, one being the pharynx proper and the other, which lies ventrally, being

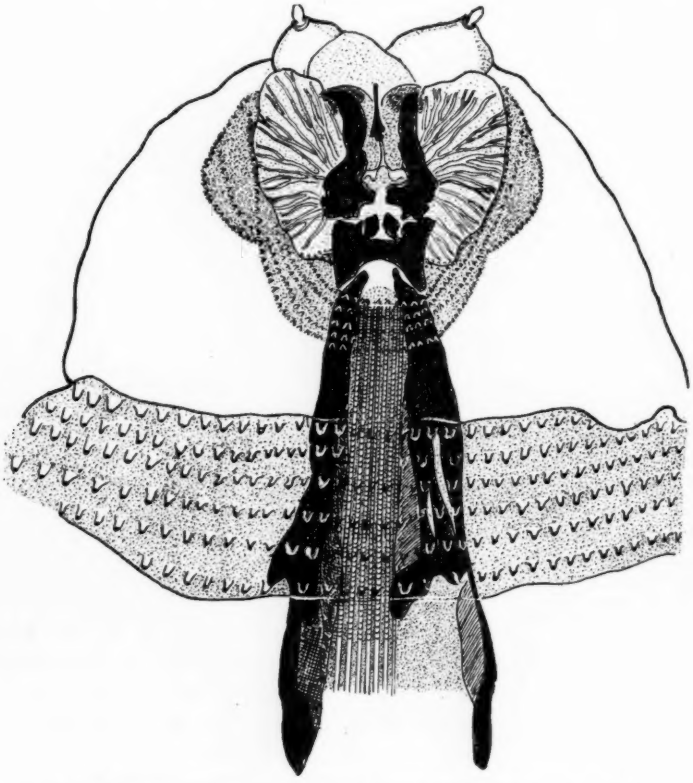

Fig. 1. *Calliphora erythrocephala* showing the pharyngeal ridges on the ventral side of the pharynx of the larva.

formed by the horizontal limbs of these ridges, constituting definite compartments or channels, and eight of them, distinctly separated from each other, are present in *Calliphora* larvae.

What purpose is served by their presence has not yet been determined. Their situation on the floor of the pharynx naturally leads one to suppose that they may act as a sort of filter, but the nature of the process of filtration has not yet been settled. These ridges divide the pharyngeal space into two

distinct, dorsal and ventral compartments. Whether the ingested fluid passes along both these channels or along only the dorsal or the ventral one, is a point which has been much debated by Wahl (1899), Wilkinson (1901),

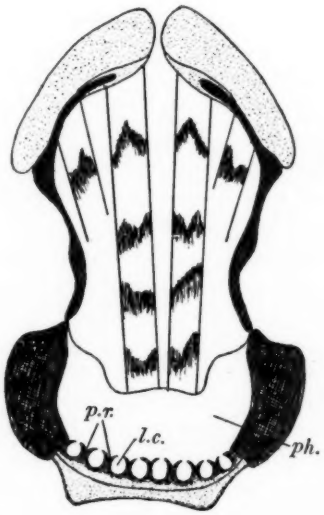

Fig. 2.

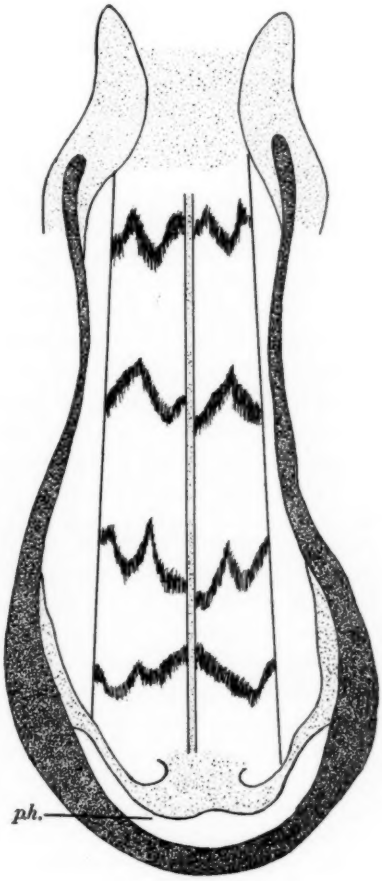

Fig. 3.

Fig. 2. *Calliphora erythrocephala* (larva) transverse section of the pharynx (*ph.*) showing the pharyngeal ridges (*p.r.*) and the longitudinal channels (*l.c.*) where the solid particles are usually caught.

Fig. 3. *Gastrophilus intestinalis* transverse section of the larval pharynx showing the absence of ridges on the floor of the pharynx. *p.h.* pharyngeal cavity.

Becker (1910), Krüger (1926) and Hennig (1935). Krüger thinks that in *Eristalis tenax* the food is diverted through both the dorsal and ventral compartments in the pharynx. Due to the action of the musculature of the pharynx its lumen becomes contracted and the sphincter opposite the

oesophagus is closed. The fluid being thus pressed forward enters into the channels formed by the T-ribs, consequently the water can pass only through the channels formed by the ridges, while the larger particles are caught by the fringes of the ribs and then pressed back into the oesophagus. The construction of the pharynx and its musculature, according to Hennig, is essentially the same in all *Cyclorrapha* larvae, whether with or without a filter apparatus. From this he concludes by saying, "Aus alle diesem kann man meines Erachtens nur den Schluss ziehen, dass der Nahrungsstrom von der Mundhole aus nur in die von den T-Rippen begrenzten Kanale gelangen kann, und dass sein Weg in den Pharynx zwischen den Filterborsten hindurchführt."

Thus it is apparent that there is a divergence of views regarding the true function served by the ridges. While many believe that they are connected with the act of filtration, there are some (Tzagardh as cited by Krüger, Hewitt) who have suggested that they are separate channels for the flow of saliva and probably some process of digestion takes place within these channels.

No experimental proof has been adduced to support any of these hypotheses.

On the other hand, if the larva restricts itself to the ingestion of liquid or semi-liquid food, as has been commonly supposed, no filtration would be necessary. Gueynot (1907) fed some *Lucilia* larvae on particles of charcoal and was able to detect only the microscopic particles in their alimentary canal and thus was convinced that they are capable of ingesting only liquid or very nearly liquid food.

In serial sections of the pharynx of a *Calliphora* larva one can see that the buccal cavity is quite broad, oval and smooth along its inside. This is followed by the pharynx which can at once be recognized by the ridges, and in the first row of them encountered they are as long, stout and chitinized as those found in the middle of the pharynx, though the individual channels become slightly larger in the latter situation; and further posteriorly they become gradually smaller and then entirely disappear.

In the following experiments larvae of *C. erythrocephala* have been used. They had been starved previously and a batch of them was fed on finely powdered charcoal presented to them in a dry state. Subsequent dissections of the alimentary canal showed that no powder had been ingested. But when the larvae were left in a Petri dish containing the powdered charcoal well mixed with some water and made up into a sort of thick fluid, the powder could be easily detected in the intestinal diverticula, proventriculus and intestine. In order to form an idea as to the largest size of these particles they were capable of ingesting, it was necessary to strain the powdered charcoal through muslin of different textures. When their alimentary canal was examined it was noticed that only the so-called microscopical particles could get into their intestine. But when the pharynx of these larvae, which had been previously fed on powdered charcoal as has been mentioned above, was dissected and examined under the low power of the microscope, the ridges were found studded with black particles of carbon, and with the high power they could be

distinctly noticed to lie inside the channels formed by the ridges. Each individual particle looked quite small in size, and they had blocked the lumen of the channel completely for some distance along its length. Sometimes only a small accumulation was found here and there. It was not unusual to encounter all the eight channels being blocked in this manner by small particles of charcoal. The constant presence of these solid particles inside the diverticulum, proventriculus and intestine of these larvae whose pharyngeal channels were found thus blocked, led one to suppose that the larger particles were held back inside these channels, allowing only the comparatively small particles to get through into the diverticulum and intestine.

A much better view was afforded in transverse sections. The carbon particles could always be seen to fill up the cavity of these channels, while the actual pharyngeal cavity always remained empty, and no such particles could ever be seen to lie inside the dorsal compartment of the pharynx. In serial sections it was noticed that the buccal cavity contained a mass of these solid particles, which probably on account of their large size were not capable of being ingested or that all the channels had already been blocked. Posteriorly, where the chitinous ridges were quite small and not long enough to enclose the channels completely, these solid bodies were found lying inside the lumen of the pharyngeal cavity as well. This showed that the solids do not find their way straight into the pharyngeal cavity, but have to pass through these channels formed by the ridges into which they are diverted from the buccal cavity.

That the suction action of the dilator muscles of the pharynx is not able to dislodge these particles caught in the channels and to force them to enter into the actual pharyngeal cavity was also determined. Some of the larvae which had been fed on carbon particles were starved till they had pupated, and when the pupae were dissected out of the puparium and the larval pharynx of puparium exposed, it was found that these solid particles were still lodged in the same situation. Thus these channels, once blocked by solid bodies, remain similarly blocked throughout the rest of the life of the larva. When adult flies were dissected soon after their emergence, the presence of the carbon particles could always be detected in the intestine and rectum, none being found in the pharynx and crop.

But notwithstanding the fact that these channels have been blocked, at least a majority of them, they are still capable of taking in their usual nourishment. This was demonstrated by feeding some larvae with charcoal and then on putrified meat which was coloured red with a watery solution of eosin. After some time they were dissected and the presence of the coloured food could easily be detected in the proventriculus and intestine, even in those larvae in which all the eight channels had been noticed to have been completely blocked with solids.

This observation undoubtedly bears out the fact that the complete blocking of all the channels does not interfere with the ingestion of the food. It may

lead one to suppose that possibly the course followed by solids and liquids in the pharynx is different. While the solid particles undoubtedly pass through the eight ventral channels the liquid probably finds its way through the dorsal compartment. In other words, one must suppose that a selective and voluntary control is exercised by them in regard to the aspiration and destination of the ingested foods as is the case in mosquitoes where the blood is generally despatched to the stomach while other fluids, particularly those containing sugars, are transferred to the diverticula. Kadletz & Kusmina (1929) have stated that in mosquitoes the aspiration of fluid is not an involuntary reflex action but is definitely under the voluntary control of the insects. With the employment of the "unsheathed proboscis" method Macgregor (1931) has demonstrated the selective control of feeding. He also found that under normal conditions only fluids of a high protein content can be made to enter the stomach directly, and other fluids would seem invariably to be stored first in the diverticula. But the following observations do not lend support to any belief that a similar selective and voluntary control is exercised by Diptera larvae.

A large number of larvae was previously starved and then fed on coarse powdered charcoal as before. After an interval of 2–3 days, when they were starved again, they were fed on particles of carmine mixed with water. Examination of the pharynx revealed that the carmine particles were also lodged in the ventral channels where the carbon particles were already present. But the distribution of the carmine particles showed that while most of them were deposited anteriorly in front of the carbon particles, the latter having completely blocked the lumen, the red carmine could in all instances be found inside the diverticulum. This proves that apparently both the large and the small particles of solids remaining suspended in the liquid or fluid medium which are ingested by the larvae are swept backwards in a current of fluid which passes through the ventral channels; the small particles easily find their way into the proventriculus while the large ones are caught in the channels, and in this way a filtration is effected. It forms a sort of dam which holds back the large solid particles only.

From what has been shown it seems reasonable to assume that both the solids and the liquids have a common direction in the pharynx and pass through the eight channels formed by the T-ribs. It is also quite unlikely that when the pharynx is compressed due to the contraction of its muscles the dorsal channel would remain patent. When the pharynx contracts this space is probably obliterated. In those larvae which are completely devoid of T-ribs the probable mechanism whereby food passes through this tract of the alimentary canal is the same as in those which are provided with these ridges. A wave of contraction passes along the pharynx, the contraction of the pharyngeal muscles obliterates the dorsal space, the fluid being pressed along the ventral wall of the pharynx and thus gains entrance into the intestine.

The reason for the total absence of the ridges in the pharynx of carnivorous

larvae as *Graphomyia maculata* and of *Calliophris riparia*, as was first observed by Keilin, can be suggested. During the process of evolution of animals, great modifications in the shape and structure of various organs have taken place according to their habits and environments. Thus, as the parasitic larvae have restricted themselves to liquid food only, it is natural to suppose that the ridges would serve no useful purpose and thus have completely atrophied and even disappeared. In saprophagous larvae, on the other hand, one can see that they have more access to solids along with liquid food and so it is in them that they are so well developed and form conspicuous structures in the pharynx.

Thus the function of the ridges seems to be connected with filtration in the nature of the separation of the large particles of solids from the liquid food, and the former are lodged in the spaces enclosed by the ridges and remain there during the rest of the larval period. The observations already reported do not lend support to any possible suggestion that the solids are caught in the pharynx with a view to their being slowly digested.

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THE PHYSIOLOGY OF DIGESTION IN LARVAE OF *GASTROPHILUS EQUI*

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Introduction

Regarding the digestive processes of different insects our knowledge is meagre. Uvarov (1928) has published an exhaustive summary on the subject. Wigglesworth (1927) investigated the process of digestion in the cockroach and found that this insect secretes enzymes by means of which it is capable of digesting practically all kinds of food substances, protein, carbohydrate, fat and various sugars. In a strictly blood-feeding insect, such as *Glossina*, the presence of proteolytic enzymes only was detected by the same author (1929). Hobson (1931), working with larvae of *Lucilia serricata*, found that the intestines of these larvae contain enzymes whereby they are capable of digesting proteins, fats and carbohydrates.

The presence of the larvae in the stomach

When the inside of the stomach of a horse containing these larvae is examined, a very heavy infestation may sometimes be met with. A lightly infested stomach contains about twenty to thirty individuals and a heavily infested one may even contain over 500. Dislodgment of these larvae by fingers is quite easy. They anchor themselves to the mucous membrane by means of the oral hooks. When they are removed, a small circular pit is left at the spot of infestation and the mucous membrane does not bleed at all. When a larva which has been removed from the stomach is placed on a piece of

linen, blotting paper, or any other soft surface, it would at once fix itself to such articles, even to the outer surface of the stomach and remain attached till it is dead. If a portion of the infested part of the stomach is cut out with the larvae still left attached to it and left on the table for some days, they make no attempt to leave the stomach wall. Under such conditions they do not live longer than 5 days. On the other hand, they can be kept alive for quite a long time if they are kept in ordinary tap water and the water is changed once a day.

For the purpose of studying the enzymes in *Gastrophilus* larvae, the insects were dissected in salt solution on a paraffined tray; the different organs were then carefully freed from their attachments to tracheae and fat bodies and then removed and placed in a drop of salt solution on a hollow slide. The order that was followed in removing the various organs was first the glands, then the different parts of the alimentary canal in the same larva.

A very small quantity of thymol was added with the idea that it would prevent any bacterial growth. The emulsion was prepared by chopping the different organs, after they had been removed, with the aid of a glass rod. In the experiments reported upon, fresh emulsions were prepared soon after dissection in order to prevent any change taking place in the properties of the enzymes.

The methods of detecting the enzymes were essentially the same as those adopted by Wigglesworth (1929) and by Tate (1929).

The buffer solutions were prepared with 0·2 *M* Na_2HPO_4 and 0·1 *M* citric acid according to McIlvaine's standards.

The larvae of *Gastrophilus* had been collected from inside the stomachs of slaughtered horses.

Alimentary canal of the larva

Mouth and pharynx. The mouth is small and is almost a circular slit which opens on the ventral surface of the pseudo-cephalon. From the characters of the mouth-parts one can say that they are best adapted for sucking liquid food.

The mouth leads into the pharynx, which is in no way different from that found in other muscoid larvae. It is entirely surrounded by the cephalo-pharyngeal armature and is attached to the interior of the ventral part of the skeleton. The space between the pharynx and the lateral blades of the pharyngeal armature is occupied by retractor muscles. The dilator muscles are attached to the dorsal parts of the pharynx and diverge as they become inserted into the dorsal and interior surface of the pharyngeal blade. A section of the pharynx shows that the ventral chitinous hooks, which are so prominently developed in saprophagous Diptera larvae, are totally absent in *Gastrophilus*.

Oesophagus. The pharynx is continued into the oesophagus and extends up to where the proventriculus begins. It is of the nature of a fine membranous tube and, before joining the proventriculus, penetrates through the fused cerebral and thoracic ganglion, and passes through the pharyngeal nerve ring.

It is devoid of any diverticula or food reservoir. *Proventriculus* or oesophageal valve prevents the regurgitation of food into the oesophagus.

Midgut. The rest of the midgut is long and is continued as a long coiled and wide tube up to where the hindgut begins. Towards its posterior part two malpighian tubes are attached and soon after their origin each tube branches into two. Just posterior to the attachment to the malpighian tubes the calibre of the intestine is abruptly narrowed; it is at this constricted part that the hindgut begins. Nearly three to four convolutions of this part of the intestine are noticed.

Hindgut. Its calibre is apparently a little smaller than the midgut, also its length. It practically makes a complete circle and then extends posteriorly in almost a straight line to the anal opening. There is no trace of any rectal dilatation.

Salivary glands. All muscid larvae possess proportionately long and elongated salivary glands, and in shape and in size are entirely different from those found in the adult condition. They project posteriorly as two white sac-like organs. The secretory duct is quite small in calibre and after the two ducts from the two glands have met, a common duct is formed which passes anteriorly and opens on the floor of the pharynx of the larva in its anterior part.

Contents of the alimentary canal

Dinulescu (1932) believes that they feed on the blood of horses and is of the opinion that digestion of blood takes place in the intestine rather slowly. The larvae do not gorge themselves with blood to the same extent as other haematophagous insects, like mosquitoes, *Stomyxis*, *Glossina*, etc.; and he never came across any specimen where he could find a great quantity of blood in its alimentary canal. According to him the blood remains always in the anterior and middle part of the intestine; the rest of the mid-intestine and the posterior part of the gut contains a fluid of a whitish substance and sometimes it is greenish. From these observations he concluded that these larvae, in spite of their permanent abode in the intestine, take blood only at great intervals. Once they have ingested a blood meal, they do not take food for the second time till the blood already ingested has been completely digested. In order to prove it, he placed some larvae in a fluid coloured with methylene blue and found that the coloured liquid penetrated into the interior only at intervals.

On the other hand, I dissected a large number of larvae which had been kept alive for some time in tap water, but in not a single individual was I able to detect the presence of blood or any liquid resembling blood. It was thought that the failure to detect blood might have been due to the fact that the larvae, by the time they were dissected, had already digested the ingested blood. So another attempt was made to dissect out the alimentary canals of larvae as quickly as possible after the horses were killed. With the help of Mr Harpley a very large number of specimens was obtained where they were

still attached to the stomach wall of the horse, and the latter was still warm. Out of 100 larvae dissected there was no trace of any blood in any part of the alimentary canal. Examination was made not only with the naked eye but also under the microscope in the fresh condition, and also when the slides were subsequently stained. No cellular structure of the blood, either a white or a red cell, was found.

Hydrogen-ion concentration of the alimentary canal

Kowalevsky (1889) adopted the method to determine the hydrogen-ion concentration of the gut of mixing litmus with the food of muscoid larvae. Weinland (1906) also followed the same procedure with *Lucilia* larvae. Wigglesworth (1927, 1929) made rough estimations of *p*H by opening the gut upon a waxed tile and adding a minute drop of indicator. Hobson (1931) added different indicators to the food of larvae and observed the colour changes along the gut. In the case of *Gastrophilus* larvae the animals were first carefully washed, its body was laid open on a waxed tile, the body fluid was next carefully washed off with distilled water and parts of the gut were cut off with a pair of scissors. They were laid separately on different waxed tiles and a watery emulsion was prepared with a minute drop of distilled water. A waxed glass rod was used for mixing the contents of the gut with the water. The rest of the process was similar to that adopted by Wigglesworth (1927). The different values thus obtained have been given below:

	Salivary glands	Pro-ventriculus	Middle part of midgut	Hindgut
In the fresh condition	7·1	7	7·4	6·8
After prolonged starvation	7·1	7	7·4	6·8
*p*H of body fluid in fresh condition				6·5
*p*H of body fluid 1 month after the larvae were kept alive in tap water ...				5·5

The values were determined with the help of B.D.H. "Capillarator".

Enzymes in *Gastrophilus* larvae

Before proceeding to the description of the experimental work, a list of the enzymes which were investigated will be arranged in more or less arbitrary groups, which can be conveniently taken up for presenting the experimental results:

Proteolytic enzymes.
Lipolytic enzyme, lipase.
Carbohydrases, such as invertase, lactase, and maltase.

The experiments for the purpose of detecting the presence of peptidase (Wigglesworth, 1927; Hobson, 1931) could not be carried out for the reason that neither of the two substances, glycyltryptophane and glycylglycine, could be obtained.

Enzymes in salivary glands

For the purpose of detecting pepsin-like enzymes, the only method employed was to observe the behaviour of coloured fibrin in the presence of a saline emulsion of salivary glands. The glands were carefully removed to avoid any injury to them during the process of dissection. The fibrin was coloured with carmine in the way described by Cole (1920).

Exp. 1. Detection of pepsin by carmine fibrin:

To a small tube A were added 0·15 c.c. or the whole of a saline emulsion of the salivary glands of five larvae, 0·15 c.c. of a buffer solution *p*H 2·5, a few tiny particles of stained fibrin and a few drops of toluol.

To another tube was added 0·15 c.c. of a saline emulsion of the glands of larvae which had been autoclaved at 120° C. for 1 hour, and the rest of the substances was the same as in tube A. This acted as a control.

The two tubes were incubated at 37° C. for 48 hours. There was no difference between the colour of the mixtures in tubes A and B, and hence there was no hydrolysis of fibrin at *p*H 2·5, indicating that pepsin is absent in an emulsion of the salivary glands.

The above experiments were repeated with an emulsion of ten glands, with a view to detecting its presence in a more concentrated strength. In other experiments the *p*H varied from 1·5 to 3·5, but the results obtained were the same, namely, that the salivary glands do not possess a proteolytic enzyme which can act in an acid medium.

Trypsin

For the purpose of detecting the presence of trypsin, the method of hydrolysis of casein and gelatine was employed. In this method an alkaline solution of casein or a solution of gelatine is incubated with the enzyme solution, and from time to time samples are withdrawn and tested for a precipitate by adding a drop or two of 0·1 per cent solution of acetic acid. As long as unaltered casein or paracasein is present, the casein is hydrolysed to peptones and finally to amino acids, which give no precipitate with the addition of acetic acid. It is true that both pepsin and rennin can cause a certain amount of hydrolysis, but not beyond paracasein, and this would serve to distinguish the action due to trypsin on the one hand and pepsin and rennin on the other. Further, these two groups of enzymes differ in the reaction of the medium at which they are most active; thus pepsin acts best in acid medium about *p*H 1·5–2 and trypsin in an alkaline medium, the optimum being *p*H 8.

Exp. 2. Action of an emulsion of salivary glands on casein solution:

A saline emulsion of salivary glands of five larvae was taken in a small tube, to which was added an equal quantity of 2·5 per cent of casein solution, an equal quantity of buffer solution of *p*H 8 and a few drops of toluol. The tube was closed by a rubber stopper and incubated at 37° C. for 24 hours.

In the control tube the emulsion of salivary glands had previously been autoclaved at 120° C. for 1 hour.

After 24 hours' incubation a drop of 0·1 per cent acetic acid was added, and from a

comparison of the turbidity produced it could be seen that the casein solution was not digested at all.

When the above experiments were repeated with an emulsion prepared from the salivary glands of fifteen larvae, there was no appreciable difference in the turbidity that could be observed between the active and control tubes.

When gelatine was used as a substrate in place of casein, the results showed that the gelatine remained unaltered.

The clotting of milk, to which is added a small quantity of calcium chloride, provides a general and most delicate test for the detection of proteolytic enzymes. But it cannot be overlooked that the clotting may be also caused by bacteria, or acid. As far as bacteria are concerned, a little thymol added to the emulsion is likely to check their growth. The hydrogen-ion concentration will afford a general indication of the acidity or the alkalinity of the medium.

Exp. 3. Action of an emulsion of salivary glands on milk.

0·15 c.c. of an emulsion of salivary glands of five larvae was prepared and to it was added 0·15 c.c. of milk. The milk was prepared as follows: to 1 c.c. of fresh milk, previously boiled and then cooled, were added 0·2 c.c. of a 5·55 per cent solution of calcium chloride and of distilled water 0·8 c.c.

In another tube the same quantity of emulsion from the glands of five larvae, previously autoclaved at 120° C., was taken and the same quantity of calcified milk was added to it.

In a third tube a little milk was taken to find out if it was good or not.

The three tubes were incubated in a water bath at 40° C. and from time to time the tubes were examined.

In similar experiments different numbers of larvae were used with a view to varying the strength of the enzyme while the quantity of the milk was kept constant, and the results have been shown as follows:

Number of larvae, the glands of which were used for the experiments	10	5	4	3	2
Time taken for the milk to clot (min.)	15 to 20	30 to 60	45 to 90	90	No clot up to 150

The control tubes did not show any positive result.

In all these experiments the nature of the milk had been found to be good as the contents in the third tube indicated.

The action of the emulsion on fibrin in a medium of *p*H 7 was next investigated, but even after 10 days' digestion bromine test could not be obtained.

This shows that a fresh emulsion of the salivary glands of *Gastrophilus* larvae does contain a proteolytic enzyme, which can only be detected by testing with milk.

Moreover, when the number of glands used for different experiments was varied, the time taken for the milk to clot was also in inverse proportion to the number used. These experiments had been repeated a number of times and in every case positive results were obtained.

It has not been possible to determine the nature of this proteolytic enzyme. It failed to show any result on the digestion of casein, gelatine and fibrin in

media of different hydrogen-ion concentrations, as could be determined by an increase in the acidity after digestion.

It was at first thought that the amount of the enzyme present in an emulsion prepared from five larvae might probably be too small to give an appreciable result when it was allowed to act on casein. So an emulsion from twenty-five glands was prepared and a 5 per cent casein solution was used as a substrate in media of different hydrogen-ion concentrations from 6 to 8, and after 48 hours' digestion the increase in acidity was determined by formol titration. The results obtained were in no way more definite than those previously described. An emulsion from fifteen larvae incubated with 5 per cent casein solution for 10 days at 37° C. entirely failed to give the bromine test for free tryptophane.

Amylase

In order to determine the presence of any starch-splitting enzyme, 1 per cent freshly prepared soluble starch solution was employed. The glandular emulsion used was also fresh. An emulsion of the glands of five larvae was made up to 0·15 c.c. and to this were added 0·15 c.c. of starch solution and 0·15 c.c. of buffer *p*H 6·8. After the mixture was prepared it was tested with dilute iodine solution ($N/100$). The iodine turned blue and immediately changed into violet colour and in a few minutes the colour faded away. At other times the blue colour with iodine was not obtained at all and the immediate colour reaction was violet. It was thought that the emulsion of the glands contained a strong reducing substance of the SH group which gives a positive nitroprusside test.

It is known, on the other hand, since the work by Khemnitz (1914) that the perivisceral fluid surrounding all the organs contains amylolytic enzyme which, with the glycogen liberated by broken-up or damaged tracheal cells, gives rise to glucose.

For further experiments, therefore, immediately after the larva was split open, the body fluid was drained off and then washed with a jet of salt solution. With an emulsion of salivary glands prepared in this way, the presence of any amyloclastic enzyme could not be detected.

Other enzymes

Without going into detail about the methods employed to detect the presence of erepsin, lipase, maltase and lactase, it may be stated that they are totally absent in fresh emulsions of salivary glands.

The experiments, referred to above, will show that the salivary secretion does not possess any enzyme except a protease, which could only be detected by the milk test and, beyond the fact that a proteolytic enzyme is present, no further light could be thrown as to its nature.

Enzymes in midgut

Amylase

The experiments were conducted in the following way:

Exp. 4. Tube A: 0·15 c.c. of saline emulsion of midgut of five larvae + 0·15 c.c. of 1·5 per cent freshly prepared starch solution + buffer solution of *p*H 6·8, 0·15 c.c. = incubated at 40° C. in a water bath.

Tube B: Autoclaved glandular emulsion used as a control.

Every 15 min. a little mixture from each tube was withdrawn and boiled with Fehling's solution. It was noticed that after 45 min. there was a well-marked reduction of copper, and the mixture in the control tube showed no such reaction. Every care was taken to remove the body fluid by washing with salt solution before the intestine was dissected out.

This proved that an emulsion of the midgut (including proventriculus, first part of the intestine up to the place of constriction just behind the origin of malpighian tubes) contains a strong amyloclastic enzyme which can hydrolyse starch to maltose.

Pepsin

No trace of any pepsin-like enzyme could be detected when coloured fibrin was employed in presence of buffer solutions 2·5 and 3. After an interval of 48 hours the *p*H was brought back to 7 by the addition of 0·01 *N* sodium hydroxide, and left in the incubator at 37° C. for 10 days. At the end of this period the fibrin looked to have been completely digested and the mixtures gave well-marked bromine reactions.

This result resembles that of Wigglesworth (1927) who observed that the trypsine-like enzyme in cockroach was inactivated but not destroyed in moderate acidity.

Tryptase

The methods employed were the same as those used in the case of salivary glands.

Exp. 5. An emulsion from the midgut of five larvae was used for each experiment. The time taken for the milk to clot was taken as an index of the activity of the enzyme. It may be stated here that while in some instances well-marked clot appeared in 15–20 min., it was considerably delayed in others up to as long as 1 hr. 15 min. The reason could only be explained by the fact that the amount of enzyme present was quantitatively different in different samples.

It was noticed that the power of the clotting of milk by the ferment was not in any way affected by heat at 80° C. for 3 min.

Casein

Exp. 6. For each experiment saline emulsion of glands from ten larvae was prepared and 5 per cent solution of casein was used as substrate. The casein solution was prepared according to the directions given by Cole. The solution was then adjusted to different *p*H. For the purpose of determining the velocity of reaction in different *p*H media, the formol

titration was employed. The *p*H was determined colorimetrically before and at the end of 24 hours' incubation and the change in the reaction is shown in Table I.

Table I

Buffer solution *p*H	*p*H before incubation	*p*H after incubation	Substrate
5	5	4·9	Casein
6	5·9	5·7	
7	6·9	6·6	
8	8	7·8	
5	5·2	5·1	Gelatine
6	6·1	5·9	
7	7·1	6·9	
8	8·2	7·9	

No separate curves have been constructed to show the velocity of reactions, as they were found to be almost identical with those found by Wigglesworth. In the case of casein, the maximum velocity occurred in *p*H 7 and with gelatine in *p*H 8.

These experiments suggest that the range of maximum activity of *Gastrophilus* protease extends a little further in the acid direction than in the case of pancreatic trypsin, and in this respect it agrees with the findings of Wigglesworth and Hobson.

After the emulsion was incubated with 5 per cent casein solution for 10 days in a medium of *p*H 8, well-marked bromine test could be obtained and leucine and tyrosine crystals separated out when the filtrate was evaporated to a small bulk.

Erepsin

In order to determine its presence, an emulsion of the midgut from five larvae was prepared and to this was added 0·15 c.c. of 1 per cent solution of commercial peptone. After the addition of toluol, it was incubated at 37° C. for 5 days. At the end of this period a little of the digested fluid was withdrawn into a tube to which was added two to three drops of strong acetic acid, and then bromine water drop by drop. No change of colour could be noticed and hence it can be stated that free tryptophane bound in the peptones was not set free by the action of the ferment.

Lipase

The methods employed for the detection of a fat-splitting enzyme or lipase were mostly qualitative. The change in the reaction in the substrate was taken as an indication of the presence of fatty acids.

Exp. 7. A 10 per cent emulsion of fresh cream with distilled water was prepared and heated to 100° C. in a water bath in order to destroy all bacteria. To 9·15 c.c. of an emulsion of the midgut of five larvae were added 1 c.c. of cream mixture, a few drops of phenol red and a little of 2 per cent solution of sodium carbonate, just sufficient to give the mixture a reddish colour. In the second tube autoclaved emulsion of the gut served as a control. After 1 hour's incubation at 37° C. the colour in the active tube turned yellow, while the control tube maintained its original colour.

Avery & Cullen (1920) and Neil & Fleming (1927) studied the presence of lipase in bacteria by using an emulsion of tributyrin. In this method a 5 per cent solution of tributyrin in phosphate buffer 7·8 was used as a substrate and the lipolytic activity was determined by the change in *p*H. A rough idea of the velocity of reaction can be formed by the change in *p*H in a given time.

Exp. 8. A solution of tributyrin which is usually adjusted to *p*H 7·8 by the addition of 0·01*N* sodium hydroxide. 0·15 c.c. of a saline emulsion prepared from the midgut of three larvae, 0·25 c.c. of 5 per cent tributyrin emulsion in distilled water are well shaken before use and brought to *p*H 7·8, mixed with 0·15 c.c. of buffer solution *p*H 7·8 containing a few drops of toluol and incubated for 1 hour at 37° C. The change in *p*H was from 7·8 to 4·1 (indicator, bromo-cresol Green).

For the purpose of determining the *p*H, the B.D.H. "Capillarator" method was used.

Carbohydrases

Among the carbohydrate-splitting enzymes only invertase, lactase and maltase were studied.

For each experiment an emulsion of the midgut of five larvae was prepared to which a little buffer solution *p*H 6·8 was added. The body fluid of each larva was carefully examined before it was dissected for the presence of free glucose, and only those which were found free from it were used. The emulsion was incubated with an equal quantity of 2·5 per cent solution of the corresponding sugar solution in presence of a buffer 6·8 to which a little toluol was added. After an incubation of 10 days the presence of the corresponding ferment was tested either by Fehling's test or by detecting the corresponding osazone crystals. On account of a dearth of materials control tests could not be performed. However, it can be stated that these three enzymes were found absent in *Gastrophilus* larvae.

Hindgut. An emulsion of the hindgut of larvae tested in the same way as previously described for sacchrogenetic, proteolytic and lipolytic activities, showed complete absence of these enzymes.

Enzymes in body fluid

It has already been shown by Khemnitz (1914) that the body fluid of *Gastrophilus* larvae contains an amylolytic enzyme, while the tracheal cells are very rich in glycogen. According to Khemnitz the reducing sugar (glucose) found in the body-cavity fluid derives from the glycogen hydrolysed by the amylolytic enzyme of the blood. Several batches of larvae examined for glucose in blood gave the following results:

In the first batch of larvae obtained, the body fluid gave negative Fehling reaction. In the second batch the fluid of almost all, when tested separately, was found to reduce Fehling's solution. Among the third batch were a few which gave, however, a faint reaction, but the well-marked brick red precipitate

was a conclusive evidence of free glucose. The fourth batch also showed in a few larvae the presence of a small trace of free glucose. Among the fifth batch there was none which showed such a reaction. The tracheal cells, as had been already demonstrated by Khemnitz (1914), showed the presence of glycogen, which can be early detected by the characteristic colour change with iodine. These cells failed to give reactions for glucose.

It was assumed that the larvae, which showed the presence of free glucose in the body fluid, were more advanced in age than those in which glucose was absent. Probably the former were nearing the stage of pupation and the glycogen of the tracheal cells was gradually converted to glucose which circulated as such in the blood.

In order to find out how this change was brought about, experiments on the following lines were carried out. Unless otherwise mentioned, the body fluid of only those larvae which did not reduce Fehling's solution was collected and used for the experiments.

(*a*) Body fluid + pure glycogen = incubated at 37° C. for 1 hour: glycogen converted into glucose.

(*b*) Body fluid + a few cells from tracheal cells = incubated at 37° C. for 1 hour: presence of free glucose detected by Fehling's test; the positive reaction begins only after 30 min. of incubation and gradually increased in intensity up to 24 hours of incubation.

(*c*) Body fluid + salt solution = Fehling's solution was not reduced after 1 hour's incubation.

(*d*) A few cells from tracheal cells + salt solution = no appearance of glucose after incubation.

These experiments confirm the results obtained by Khemnitz (1914) that the body fluid of *Gastrophilus* larvae contains an amylolytic enzyme which hydrolyses glycogen added directly to or deriving from tracheal cells.

Discussion

In a strictly blood-feeding insect, such as *Glossina*, the enzymes studied by Wigglesworth were in agreement with their peculiar feeding habits; so in the case of an insect like cockroach and *Lucilia* larvae. In an insect like *Glossina* which feeds entirely on blood, one would not expect to find all the enzymes which are present in a cockroach. The presence of amylolytic, proteolytic and lipolytic enzymes in the intestine of *Gastrophilus* larvae would serve no useful purpose if their habits were restricted to feeding on blood only. On the other hand, it would agree with the view that they feed on the fluid materials which are the products of digestion present in the stomach of horses. This food material is, as one can well suppose, composed of proteins, fats and carbohydrates, and the midgut of the larvae are therefore provided with enzymes, by means of which they are capable of digesting such a food. It is

probably for this reason that the horses do not show any symptoms that they have suffered from the infestation.

Thus we find that the foregut acts as a tube for the passage of food, the salivary glands secrete only a proteolytic enzyme, the midgut amylolytic, proteolytic and lipolytic enzymes, and that the hindgut does not secrete any enzyme at all.

Summary and conclusions

The results of the study on the digestion of *Gastrophilus* larvae may be summarized as follows:

(1) Pepsin-like enzymes are totally absent.

(2) The salivary glands secrete a proteolytic enzyme, which has no effect on the digestion of casein, gelatine and fibrin, but acts on milk alone.

(3) The midgut is capable of secreting amylolytic, proteolytic and lipolytic enzymes.

(4) The body fluid contains a strong reducing substance.

(5) The body fluid, as was shown by Khemnitz (1914), contains an amylolytic enzyme which is also capable of converting glycogen into glucose.

(6) Free glycogen and glucose circulate in the body fluid of the larva during the prepupal and possibly the pupal stage also.

(7) The larva of *Gastrophilus* does not feed on blood, but lives on the fluid material partly digested by its host in the stomach.

(8) For this reason the horses do not show any symptoms even when there is a heavy infestation.

My sincere thanks are due to Prof. D. Keilin for his unfailing courtesy in helping me in every possible way in the conduction of the above investigation. I am also indebted to Dr Wigglesworth for taking the trouble to go through the manuscript. I am indebted also to Dr Tate of the Molteno Institute for his help and useful suggestions.

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THE INTESTINAL PROTOZOA OF THE WOOD-FEEDING ROACH *PANESTHIA*

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(With Plates VIII–X, containing Figs. 1–45 and Figs. I–VIII in the Text)

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INTRODUCTION

In their beautiful and complete monograph on the wood-feeding roach, *Cryptocercus punctulatus* and its Protozoa, Cleveland and his associates (1934) mention the fact that, although a wood-feeder, *Panesthia javanica* does not harbour cellulose-digesting Protozoa. When this statement was made they had in mind the typical cellulose-digesting hypermastigote and polymastigote flagellates which are so abundant in *Cryptocercus* and most termites. With the exception of one *Endamoeba* the above statement is true, and even with this single form probably little or no benefit is derived by the host, so we cannot look upon the case as one of symbiosis. However, the alimentary canal of *Panesthia* contains so many diverse forms as to rival any but its close relative *Cryptocercus*. In all there are at least 14 species of Protozoa living in association in the large hindgut of *Panesthia*, and representing 3 of the 4 subphyla of the Protozoa. Two are members of the Sarcodina; 2 of the Mastigophora; and 10 of the Infusoria.

In regard to the protozoan parasites of *Panesthia* it is interesting to note that definite comparisons can be made between the domestic cockroaches (*Periplaneta*, *Blatta*) on the one hand, and a number of the Termitidae on the other, while only 2 small flagellates are common (as to genera) to *Cryptocercus*, *Panesthia*, and *Periplaneta*. It is generally assumed that the domestic cockroaches and *Panesthia* have both been derived from common ancestors which in turn must have been similar to, if not identical with, the ancestor of *Cryptocercus*. The evolution of the protozoan parasites, commensals, and symbionts has progressed surprisingly far in some cases and has been surprisingly slow in others. So while the characteristic cockroach fauna is found intact in *Panesthia* (except for the genus *Lophomonas*) an additional group of totally different forms has evolved to live in close association with the common types. These are the bizarre ciliate types, unrecorded to date from any other host.

It is the purpose of this paper to describe as completely as possible the various Protozoa found inhabiting the alimentary canal of *Panesthia javanica* and *P. spadica*, in regard to their morphology, cytology, taxonomic status, and evolutionary significance.

MATERIAL

In connexion with his work on *Cryptocercus* Cleveland had wood-feeding insects collected for him from many parts of the world. During one of her collecting trips Miss Jane Collier, technical and research assistant in Protozoology, Hervard Medical School, brought back numerous wood-feeding roaches from Java, Bali, and China. As these were found to harbour no symbionts they were turned over to me for a preliminary examination of the rather large and peculiar ciliates found living in their hindguts. Most of the roaches collected by Miss Collier have not yet been identified. One species,

however, collected in the Philippine Islands, was *Panesthia javanica*, which was found to harbour the most varied fauna of all the wood-feeding forms, with the exception of our native *Cryptocercus*. It was decided, therefore, to confine the first of this series of studies to the Protozoa of the genus *Panesthia*, as the material was more abundant and the host known.

Many questions arose that could not be answered from fixed and stained preparations, so, thanks to the kindness of Dr L. B. Uichanco (Professor of Entomology at the University of the Philippines, Agricultural College, Laguna, P.I.), I was able to obtain a large colony of living *Panesthia javanica*. This colony was collected on the eastern slope of Mt. Maquiling, Luzon, at an altitude of between 200 and 700 m. The roaches were sent to me in rotten wood and excelsior. Some of them were left in their native wood for a long period, while others were transferred to small dishes with nothing but moist filter paper upon which to feed. Many are still alive and seemingly in perfectly normal condition after a period of 9 months.

I have had the opportunity to examine *Panesthia spadica* and to compare its fauna with that of *P. javanica* through the kindness of Dr Harold Kirby, Jr., of the University of California. During extended travelling in 1934 and 1935 he collected *P. spadica* in Japan, prepared intestinal smears and turned this material over to me. It, as well as the material collected earlier by Miss Collier, was prepared in the field as follows: the intestinal contents of the insects were smeared on cover-glasses and fixed in Schaudinn's or Flemming's fluid, washed, and then stored in alcohol for transfer. In this way 25 slides from 4 *P. spadica* were obtained.

To Dr L. R. Cleveland I wish to express my sincere thanks for calling my attention to the forms described below and for turning over to me the initial material. Also I am deeply indebted to Dr L. B. Uichanco for the living *P. javanica* without which many of the observations recorded here would have been impossible. I take pleasure in thanking Dr Harold Kirby, Jr., for the material from *P. spadica*. And finally I wish to express my appreciation to the Department of Zoology of Columbia University for affording me the facilities of their laboratory while this work was in progress.

METHODS

In addition to extensive studies from life of all the species of Protozoa in *Panesthia javanica* permanent preparations were made in the same manner as described by Cleveland *et al.* (1934) in their studies on the Protozoa of *Cryptocercus*. Various fixatives and stains were employed, among the most useful combinations being Bouin's fluid followed by Heidenhain's haematoxylin; Schaudinn's fluid followed by Heidenhain's or Delafield's haematoxylin or the Feulgen nucleal reaction; Fleming's fluid followed by Heidenhain's haematoxylin; and Zenker's fluid followed by Mallory's triple stain as described by Sharp (1914) and Yocom (1918). Destaining of Heidenhain's haematoxylin was carried out in 2 per cent iron alum; saturated aqueous picric acid (see

Kidder, 1934 *a*); 10 per cent hydrogen peroxide (see Kidder, 1933); and 70 per cent alcohol acidified with hydrochloric acid. The preparations destained in alum and peroxide were especially useful for morphological studies of the fibrillar system, while those destained in picric acid and acidulated alcohol were excellent for nuclear detail.

Sections of whole guts were made following the method of Cleveland *et al.* (1934). Individual ciliates were also fixed, dehydrated, and embedded separately, being oriented in given planes before cutting. The former method gave less excellent results than the latter, as it was found that the central portion of the gut contents usually did not remain in the ribbon. Also a knowledge of the exact orientation proved to be a great aid in reconstructing the organism from sections. The sections were cut at thicknesses varying from 3 to 7 μ.

In the material from *Panesthia spadica* prepared by Dr Kirby fixation and staining were carried out in Flemming's fluid followed by Régaud's haematoxylin, or Schaudinn's fluid followed by Heidenhain's or Delafield's haematoxylin. In some cases the preparations were counter-stained with acid fuchsin. No material from this roach was available to me either for sectioning or for a study of the organisms in life.

OBSERVATIONS

Intestinal flagellates

Of the 2 flagellates found to inhabit the hindgut of *Panesthia javanica* the most frequent in occurrence is a member of the genus *Monocercomonoides*. This flagellate has been found to occur in abundance in about 80 per cent of the *Panesthia javanica* examined. Although it is small, certain details can be ascertained in smears stained with Heidenhain's or Delafield's haematoxylin. Infrequently (so far in only 4 roaches) a member of the genus *Hexamita* is found. When present this form is very abundant, existing in as great numbers as *Monocercomonoides*, with which it is associated. In life it is not difficult to distinguish one type from the other because of the characteristic motions. *Hexamita* is bilaterally symmetrical, swims in a more or less straight line, and is metabolic. *Monocercomonoides* is slightly curved, swims in a jerky or zigzag fashion, and the body form remains relatively constant.

Both *Monocercomonoides* and *Hexamita* are very minute and it has been therefore impossible to determine the finer structures of the body exactly. I offer the following brief descriptions of these flagellates after a careful study of hundreds of individuals from numerous hosts, in order to complete the record of the Protozoa inhabiting the alimentary canal of *Panesthia javanica*. So far neither flagellate has been found to occur in *P. spadica*.

Monocercomonoides panesthiae sp.nov.

(Figs. 1–8)

In the posterior part of the hindgut of *Panesthia javanica*, in most individuals of my colony, I found a heavy infection of small flagellates belonging to the genus *Monocercomonoides* Travis, 1932. In life these flagellates are very active. The majority are elongate forms measuring from 6 to 10μ, although many are spherical, measuring from 4 to 5·5μ in diameter. These latter, I believe, are forms about to undergo binary fission. There are 3 long anterior flagella that are usually united into a stout whip with only the distal ends free. These flagella act as a single structure beating back and forth as if hinged at the body. One very long and thick flagellum is directed posteriorly but is free from the body throughout its length. This can be determined as the flagellate is turned about by the action of the anterior whip. The trailing flagellum is about one-third again as long as the anterior ones. In the elongate forms a very conspicuous hyaline axostyle, of the *Trichomonas* type, is clearly visible. Unlike *Monocercomonoides globus* (Cleveland *et al.* 1934) it extends for some distance from the posterior end of the body. In some cases it appears to be evenly pointed while in others it possesses an enlargement at its distal end in the form of an arrowhead, much the same as that described by Kirby (1931) in *M.* (*Eutrichomastix*) *axostylis* from the termite *Nasutitermes kirbyi*. I have never observed any structures corresponding to the rings about the posterior region of the axostyle found in the last-named species. Frequently granular debris collects about the protruded portion of the axostyle and is carried about by the flagellate.

In stained material the axostyle remains clear except in very heavily stained specimens. Even in these it never has the appearance of a heavily stained filament, as described in so many species. Rather it is a grey rod. One very noticeable result of fixation is the contraction of the axostyle, so that in most forms no trace of this organelle can be seen protruding from the body. Occasionally one will be preserved in a life-like condition (Fig. 5).

The nucleus is spherical, rather large, and located near the anterior end. The nuclear membrane is thick and retains the stain. Within the nuclear membrane is a clear area surrounding the chromatin, probably the result of shrinkage due to fixation. In material fixed in Bouin's fluid the chromatin appears as balls in a lightly staining plastin matrix, while material fixed in Schaudinn's fluid shows the chromatin balls much more deeply stained with the plastin largely dissolved out or rendered non-stainable.

Anterior to the nucleus is a deeply staining blepharoplast. This body sometimes appears to be lying within the anterior end of the clear axostyle, while in other cases the axostyle cannot be seen in this region. From the blepharoplast a rhizoplast is sometimes visible extending to the nuclear membrane, though it can be seen only in particularly favourable specimens. All the flagella appear to originate directly from the single blepharoplast.

In the region of the blepharoplast appears a slit-like clear area which may represent a cytostome (Figs. 2 and 5). I have never been able to see any trace of this opening in the living forms, but it occurs so often in fixed and stained preparations that I believe that it cannot be an artifact in every case. It always ends in a point near the nucleus and is thus somewhat different from the oval cytostome of *M.* (*Eutrichomastix*) *axostylis* (Kirby, 1931).

In a great many, although not all, of the stained preparations deeply staining bodies appear in the cytoplasm. In material fixed in Bouin's fluid and stained with Delafield's haematoxylin one or more irregular masses of material are found in the posterior endoplasm near the axostyle (Figs. 1–2). They appear somewhat similar to the deeply staining structures sometimes found in *Giardia*, and which have been homologized with the parabasals of other flagellates by Kofoid & Christiansen (1915). In material fixed in Schaudinn's fluid and stained in Heidenhain's haematoxylin they either do not show or appear as faint grey patches. However, with the latter technique, as well as in the Bouin-fixed material, a deeply staining sphere occurs just posterior to the nucleus. This appears in both the elongate and rounded individuals and seems to be constant in size. It is usually located near the axostyle and may represent a parabasal body. I have never been able to observe any connexion between this body and the blepharoplast, but such a connexion, if existent, would be difficult to see.

Many stages of binary fission have been seen. These seem to correspond closely to those described by Bělař (1916) for *M.* (*Monocercomonas*) *orthopterorum* from *Periplaneta*. In all cases these stages were observed in the spherical forms, the condition that precedes nuclear activity. In early division forms I have never been able to see any trace of the axostyle. It is probable that this structure is resorbed at an early stage. The division of the blepharoplast, or its active component, takes place and the two halves move apart leaving a deeply staining intradesmose between them (Fig. 6). This intradesmose is undoubtedly the "central spindle" of Cleveland *et al.* (1934). To each of the daughter blepharoplast complexes are still attached two of the old flagella. I have not been able to demonstrate the formation of the new flagella. This process appears to be very late in occurrence. The actual nuclear division proceeds as is shown in Figs. 6–8. The central spindle does not appear to compress the nucleus unless possibly at the very late telophase. During the telophase two new axostyles make their appearance, one from the region of each division centre. They are thin refractile rods, pointed towards the middle of the body, with their distal ends often crossed (Fig. 8).

Monocercomonoides panesthiae resembles most closely the form described by Parisi (1910) as *Trichomastix orthopterorum* from the domestic cockroach. Bělař (1916) placed this form in the genus *Monocercomonas*, but if we are to follow Travis (1932) it would now be called *Monocercomonoides orthopterorum*. There are a few distinct differences between the last-named species and the form from *Panesthia* which I believe to be significant. The most obvious one

is the difference in the structure and stainability of the axostyle. In *Monocercomonoides orthopterorum* the axostyle is a deeply staining filament, while in *M. panesthiae* it is a stout hyaline rod of the *Trichomonas* type. The nuclei of the two species appear to be different in the resting condition. Instead of the chromatin being clumped into a "karyosome" as described by Bělař, it is always more or less dispersed in the form of spheres in *Monocercomonoides panesthiae*. The sizes of the two species are similar enough to be disregarded. Parisi (1910) gives the range from 8 to 10μ, while Bělař (1916) states that in fixed material the range is from 3 to 7μ.

Comparing *M. panesthiae* with *M. globus* from *Cryptocercus* (Cleveland *et al.* 1934) many differences are noted. *Monocercomonoides globus* is not only larger (8–14μ) but always much broader. It has a "heavily staining, somewhat ribbon-shaped axostyle, which never protrudes from the body" (*loc. cit.* p. 305). I have had the opportunity to examine hundreds of specimens of *M. globus* from *Cryptocercus punctulatus* and I can definitely say that these two species (*Monocercomonoides globus* and *M. panesthiae*) are distinct in spite of the close relationships of their respective hosts. The deeply staining nucleolus surrounded by the lightly staining area, so characteristic of the nucleus of *M. globus*, is never found in *M. panesthiae*.

Diagnosis: Monocercomonoides panesthiae sp. nov. Subellipsoidal to spherical in shape, ranging from 3 to 10μ in length. Axostyle hyaline, rod-like, extending through the body centre and beyond the posterior end. Trailing flagellum longer and heavier than the 3 anterior. Nucleus spherical, anterior, containing scattered spheres of chromatin. Deeply staining cytoplasmic sphere just posterior to nucleus near axostyle.

Type host: Panesthia javanica from Mt. Maquiling, Luzon, Philippine Islands.

Hexamita cryptocerci Cleveland *et al.* 1934

(Figs. 9–11)

In 4 roaches from my colony I have found very heavy infections with *Hexamita*. Although the flagellate is very much smaller in average size than *H. cryptocerci* from *Cryptocercus*, it is otherwise so similar that I believe both to belong to the same species. I have examined *Hexamita cryptocerci* and I can find no morphological differences between it and the form from *Panesthia*.

The 8 flagella are disposed as described by Cleveland *et al.* (1934) in the material from *Cryptocercus*: 2 anterior, 2 caudal and 4 lateral. In stained smears these flagella may be very much twisted, but they stain well and can readily be counted. In living organisms the 2 anterior and the 2 caudal are much less active than the laterals. The organism swims in a fairly straight line, apparently through the activity of the lashing lateral flagella. The posterior laterals seem to be anchored to the body for about one-third the body length.

The length of the body in 10 living forms varied from 3 to 8μ with an average of 6·5μ. In material fixed in Schaudinn's fluid there was considerable

shrinkage and the average length was found to be 5μ. From these figures it will be seen that the fixed specimens from *Panesthia* average just one-half the length of those from *Cryptocercus*. It may be possible to account for the discrepancy by the environmental conditions found in the 2 hosts.

The 2 nuclei, typical of this genus, are elongate and lie near the anterior end of the body, one on either side. In stained preparations they appear to be made up of deeply staining homogeneous chromatin, surrounded by a clear non-staining area.

Just anterior to the nuclei are invariably seen 2 deeply staining blepharoplasts. Three flagella certainly, and probably a caudal as well, arise from each of these. I was never able to see any connexion between the nuclei and blepharoplasts. Thickened fibre-like structures, the so-called axostyles, proceed from each blepharoplast into the posterior endoplasm. At the distal end of each may be seen a deeply staining granule. That these structures are not merely the proximal ends of the caudal flagella seems certain because of their diameter. It is probable that they are rod-like structures to which the internal portions of the caudal flagella adhere, because when one or both is curved within the endoplasm there is no evidence of the caudal flagella being pulled away.

The *Hexamita* from *Panesthia* differs from *Hexamita periplanetae*, described by Bělař (1916), mainly in the disposition of the flagella. The anterior and laterals of the former are similar to those of *Giardia*. This fact was mentioned by Cleveland *et al.* (1934) as possibly being a specific feature. The caudal flagella are always very close together, while in the other species of *Hexamita* they are far apart. (See, however, Cleveland *et al.* for a discussion of the figure of *Hexamita intestinalis* given by Wenyon, 1926.)

It must be borne in mind that, although these flagellates lend themselves very well to the ordinary staining techniques, any observations on the minute details of structure must be provisional. In an organism so small (5μ) one must not expect to be able to make out all the details necessary for a complete account.

I have never observed any clear cases of division on my slides although I have seen a number of forms with 4 nuclei, similar in every respect to the quadrinucleate forms described by Cleveland *et al.* (1934).

Intestinal amoebae

Three species of amoeba have been encountered in the hindgut of *Panesthia javanica* and one of these was found in a specimen of *P. spadica*. Two undoubtedly are species of *Endamoeba*, while the third I have been unable to identify. The largest *Endamoeba* was found in about 50 per cent of the *Panesthia javanica* examined, the smaller species in about 10 per cent, while the unidentified species was found in only one roach. In no case was the infection heavy, but the number of the larger *Endamoeba* was sometimes such

that a single coverglass was found to contain as many as 50 specimens. Cysts of all 3 species were found, but never in abundance.

Endamoeba philippinensis sp.nov.

(Figs. 12–15)

This species occurs rarely (in about 10 per cent) in the hindgut of *Panesthia javanica*. It is small, ranging from 8 to 13μ in length and from 6 to 9μ in width. The pseudopodia are hyaline and usually only 1 is formed at a time, although occasionally 2 or 3 are seen. There is no sharp differentiation between endoplasm and ectoplasm except in the pseudopodia. The movements of the organism are characteristic. A pseudopodium is rapidly formed on one side and the endoplasm flows into it. After a pause another pseudopodium is formed usually on the opposite side, and the organism moves back. No appreciable progress is made but the amoeba is constantly shifting back and forth.

The nucleus can usually be distinguished in life and stands out sharply in stained preparations. It is spherical and closely resembles that of *Entamoeba coli*. There is a single compact endosome (karyosome), eccentric in position, from which radiating lines run out to the nuclear membrane. The chromatin is in the form of a thin shell a little distance in from the nuclear membrane, which in optical section appears as an even ring of spherical granules. I have never encountered any phases of division.

The endoplasm is granular and is filled with food vacuoles containing bacteria. I have never detected any wood in these vacuoles.

Cysts have been found containing 1 (Fig. 14), 2 (Fig. 15), and 4 nuclei. The cysts measure 7–8μ in diameter. They are spherical and possess a thick wall. Their nuclei are exactly like those of the trophozoites. In most of the bi- and quadrinucleate cysts deeply staining irregular chromatoid bodies are found, varying in number from 1 to 4. I have never seen such bodies in uninucleate cysts.

The nucleus of this species is obviously of the "*Entamoeba*" type and differs greatly from that of the type species of *Endamoeba* (*E. blattae*), therefore it probably should be placed in another genus (perhaps *Poneramoeba* Lühe). Several similar amoebae have been described from insects, such as *Entamoeba minchini* (*Löschia hartmanni*) from *Tipula* larvae (Mackinnon, 1914), *Entamoeba belostomae* from the water-bug (Brug, 1922) and *Entamoeba apis* from the bee (Fantham & Porter, 1911). However, until the nomenclature of these forms is clarified further I prefer to refer the species described above from *Panesthia* to the genus *Endamoeba*, recognizing its uncertain status. (For a discussion of this question the reader is referred to Kirby, 1927.)

Diagnosis: Small amoebae (8–13 × 6–9μ), ellipsoidal to spherical, and feeding on bacteria. No clear differentiation of ectoplasm. Pseudopodia broad, usually formed one at a time. Nucleus spherical, containing a definite eccentric

endosome and regular chromatin granules situated a little distance inside the nuclear membrane. Cysts spherical, containing 1, 2, or 4 nuclei, and from 1 to 4 chromatoid bodies. Cyst wall thick.

Type host: Panesthia javanica from Mt. Maquiling, Luzon, Philippine Islands.

Endamoeba javanica sp.nov.

(Figs. 16–20)

This species occurs frequently (in about 50 per cent) in the hindgut of *Panesthia javanica*, and was found in 1 *P. spadica* out of 4 examined. It varies greatly in size, the majority being from 20 to 45 μ in length and from 15 to 25 μ in width. One was found measuring 121 by 78 μ and another 88 by 54 μ, but these were exceptional.

In life this form is easily recognized by the immense amount of wood and cellulose fibres in its endoplasm. Because of these inclusions the nucleus is usually obscured. There is a rather thick and transparent ectoplasm, free from wood particles, usually thrown into numerous folds. In life many sluggish pseudopodia always project from the body, but little movement is noticeable. In fixed material the smaller pseudopodia usually have contracted so that only the broader ones remain.

The nucleus, as seen in fixed and stained preparations, is somewhat similar in appearance to that of *Endamoeba disparta* from the termite *Mirotermes hispaniolae* as described by Kirby (1927). The chromatin is concentrated in a loose mass near the centre, where, in sufficiently destained specimens, an endosome can usually be seen. The chromatin reacts definitely though faintly with the Feulgen reagent. The nuclear membrane is exceedingly thick and generally slightly folded. Adhering to its inner surface are varying numbers of haematoxylin-staining bodies (Figs. 17, 20). These bodies are irregular in shape, number, and occurrence and are probably homologous with the nucleoli of *Endamoeba disparta* (Kirby, 1927) and *E. beaumonti* (Kirby, 1932). Between the nuclear membrane and the chromatin mass is a fibrous reticulum on which are flocculent lightly staining masses. After the Feulgen reaction this area remains uncoloured.

A few mitotic stages have been found. Fig. 18 represents what is probably a prophase with the chromatin aggregated into spherules, and what appears to be an achromatic figure originating in a deeply staining granule at the nuclear periphery. This stage is very similar to the corresponding one of *Endamoeba beaumonti* (Kirby, 1932). Fig. 19 represents an organism with 3 nuclei, 2 of which are decidedly smaller than the third. This has probably resulted from the division of one cystic nucleus and not the other forming the *Nnn* condition described by Cleveland & Sanders (1930) in *Entamoeba histolytica* and by Sanders (1931) in *E. ranarum*. I have observed many cases where 2 equal nuclei were present, perhaps representing metacystic stages.

A few cysts have been seen both in life and in fixed preparations. They are

uniformly spherical, 16–22 μ in diameter, and the cytoplasm is devoid of wood. The cyst wall is extremely delicate and difficult to observe. Most of these cysts are uninucleate, but occasionally binucleate ones have been found (Fig. 20). The nucleoli are much more apparent in the nuclei of the cysts than in those of the trophozoites, but otherwise they are similar. No chromatoid bodies have been observed.

Xylophagous amoebae are rare, being reported hitherto only from some of the termites (Kirby, 1927). Because of its habits and its nuclear structure *Endamoeba javanica* resembles most closely, as stated above, *E. disparta* (Kirby, 1927). I have had the opportunity, through the kindness of Dr Kirby, to examine preparations of that species, and I am convinced that the two forms are different. The average sizes are similar (*E. javanica* $31 \times 20\,\mu$; *E. disparta* $32\,\mu$ in diameter), but organisms with fusiform nuclei, occurring as frequently as those with spherical nuclei in *E. disparta*, have never been found in my material. Also the absence of cysts in *E. disparta* is another significant difference. The nucleus of *Endamoeba beaumonti* from *Amitermes* (Kirby, 1932) is similar to that of *Endamoeba javanica*, except that its nucleolus is more compact and more deeply staining. *E. beaumonti* is not xylophagous, however. Similar conditions are found in *Entamoeba thomsoni* from the domestic cockroaches, in regard to the nuclear make-up (Lucas, 1927); but this species differs from *Endamoeba javanica* in a number of other details, including shape, ectoplasmic differentiation, and food habits.

Diagnosis: Shape irregularly ellipsoidal to spherical. Average size $31 \times 20\,\mu$. Xylophagous. Clearly defined ectoplasm. Many very sluggish pseudopodia, small and thin to large and blunt. Nucleus spherical, sometimes indented. Nuclear membrane thick. Chromatin concentrated near the centre. Endosome present within the chromatin mass. Nucleoli, number varying, on inner surface of nuclear membrane. Cysts spherical, measuring from 16 to $22\,\mu$ in diameter, containing 1 or 2 nuclei. Cyst wall delicate.

Type host: Panesthia javanica from Mt. Maquiling, Luzon, Philippine Islands. (Common.)

Additional host: Panesthia spadica from Gifu, Japan. (Rare.)

Amoeba sp.

(Fig. I)

In one specimen of *Panesthia javanica* that had been kept on a diet of filter paper for 3 months there occurred, in addition to the 2 species of *Endamoeba* and the normal ciliate fauna, an amoeba that I have been unable to identify. Many of these organisms were observed in life, and slides prepared from the intestinal contents contained several individuals.

This amoeba is elongate, measuring from 30 to $35\,\mu$ in length and from 16 to $20\,\mu$ in width, and moves with great rapidity. One or two clear pseudopodia are protruded and the endoplasm flows immediately into them. The

organism is very similar in appearance to the small free-living amoebae in the scum of pond water. Its endoplasm is clear but contains numerous food vacuoles filled with various inclusions. The nucleus is plainly visible in life and looks very much like the nucleus of *Amoeba dubia*. In stained preparations (Fig. I A) it appears filled with chromatin spherules and occasionally larger deeply staining masses are present.

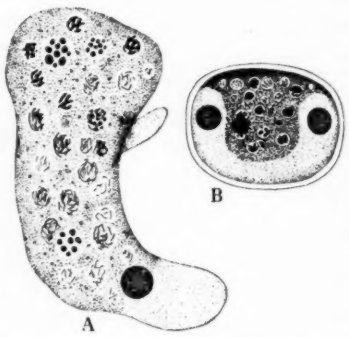

Fig. I. *Amoeba* sp. A, trophozoite. B, cyst. Schaudinn-Heidenhain's haem. ×1000.

Cysts of a peculiar type were prevalent in this one roach. They are ellipsoidal structures (measuring from 20 to 25μ in length) with a definite cyst wall. The cytoplasm is divided into two areas, a coarsely granular one containing food inclusions and an elongate finely granular one containing the two nuclei. The nuclei are placed at either end of the cyst (Fig. 1 B) and are like those of the trophozoites. The cyst is transparent in life and its nuclei plainly visible.

My observations on this form are incomplete, as the material was limited. It may be that this amoeba was an accidental inhabitant of this roach, but the fact remains that it was able to exist in the trophic state, like *Vahlkampfia punctata* as observed by Chatton & Lalung-Bonnaire (1912) in the human intestine, and *Vahlkampfia* sp. described from the intestine of the crane-fly larva by Mackinnon (1914).

Intestinal ciliates

It is not surprising to find the genus *Nyctotherus* represented among the intestinal inhabitants of *Panesthia*. Numerous species of this genus occur in a variety of hosts, both vertebrate and invertebrate. *Nyctotherus ovalis* occurs regularly in great numbers in most individuals of *Periplaneta*. It is also to be expected that different species would occur in a host offering such a peculiar type of environment as is found in a wood-feeder. *Nyctotherus* is found in at least 2 termites, according to Dobell (1910) and Kirby (1932). In the case of *Nyctotherus silvestrianus* from *Amitermes*, reported by Kirby, the general resemblance to *Nyctotherus ovalis* is marked. This resemblance is also seen in the species from *Panesthia*.

What is surprising, however, is the occurrence of a large number of peculiar ciliates that make up the most conspicuous part of the intestinal fauna. These form a compact taxonomic group possessing structural resemblances to *Nyctotherus* but differing from the latter in shape, and particularly in the position of the mouth. The group is peculiar to wood-feeding cockroaches, as far as is known. Its members I have placed in two new genera: *Clevelandia*

gen.nov., and *Paraclevelandia* gen.nov., in honour of Dr L. R. Cleveland who first described the Protozoa of wood-feeding cockroaches. It has been necessary to create a new family for these two genera (Clevelandiidae fam.nov.). An account of the various ciliates found in the hindgut of *Panesthia javanica* and *P. spadica* follows.

Nyctotherus uichancoi sp.nov.

(Figs. 21–23; Figs. II, III, and VIII H)

In *Panesthia javanica* and *P. spadica* a *Nyctotherus* is found which, because of certain structural peculiarities described below, I believe to be a new species. I am calling it *N. uichancoi* in recognition of the kindness of Dr L. B. Uichanco in sending me living *Panesthia*. About 90 per cent of all the individuals of *Panesthia javanica* were found to harbour from 20 to 100 specimens of this species of ciliate in the hindgut. In many it was more prevalent than all the other ciliates combined (see Table on p. 189). On the other hand, my material of *P. spadica* was very lightly infected, yielding only 6 specimens from 4 roaches.

Nyctotherus uichancoi is slightly flattened dorsoventrally. The anterior end is usually evenly rounded but occasionally tapers slightly as in *N. ovalis*. This is especially true of the very small forms which probably represent newly excysted individuals. The posterior end is usually rounded.

One hundred individuals chosen at random gave the following measurements: average length 79 μ (26–145 μ); average width 62 μ (15–91 μ). About 85 per cent of these individuals were large trophozoites and averaged 106 μ in length by 77 μ in width. The very small "excysted forms" were few, but their reduced size lowered the average.

The peristome begins slightly below the anterior end and follows the right lateral margin to about the middle of the body, where it opens into a very large cytostome. The cytopharynx continues into the body in a nearly transverse direction for approximately one-third of the body width, where it curves obliquely towards the posterior end. The distal end recurves in a slight spiral until it points antero-ventrally. The degree of curvature varies in different individuals. In small forms the amount of spiralling is reduced, and the distal end of the cytopharynx may be pointed posteriorly (Fig. 23). From the end of the cytopharynx a tube (Fig. II A, *cyt.t.*), a continuation of the walls of the cytopharynx, extends for a short distance, ending blindly in the endoplasm. This is undoubtedly homologous with the "pharynx" in *N. ovalis* as described by Ten Kate (1927) and probably with the "continuation tube" of Higgins (1929) in *N. cordiformis*. It is probably likewise homologous with the "slender, band-formed structure" of *N. silvestrianus*, described by Kirby (1932), although not so extensive.

The body surface is ridged longitudinally, and between the ridges emerge the peripheral cilia. The ciliary rows originate in a suture anteriorly and pass dorsally and ventrally towards the hind end, where they converge in an irregular

oblique suture on the dorsal surface. This posterior suture does not appear to be formed from a fibre but is rather the configuration produced by the combining of the ciliary rows. It appears that the co-ordinating fibres unite in this region as in *Conchophthirius anodontae* (Raabe, 1933; Kidder, 1934 *b*), forming an irregular network.

The membranelles of the peristome begin at the right end of the anterior suture, and their basal plates are all connected by a longitudinal fibre which

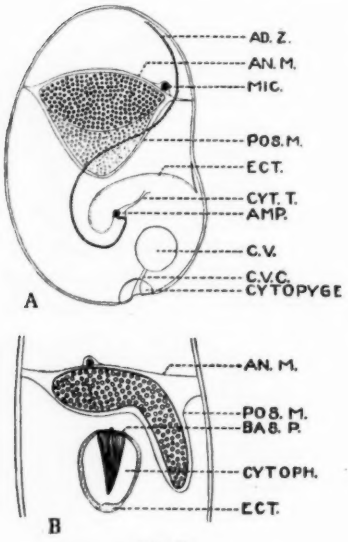

Fig. II.

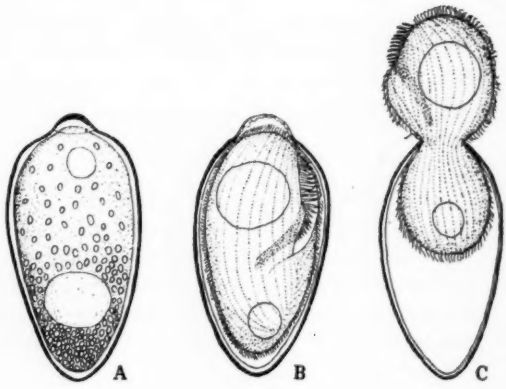

Fig. III.

Fig. II. Diagrammatic representation of *Nyctotherus uichancoi*. A, dorsal view. B, right lateral view in section. Abbreviations: *Ad.Z.* adoral zone in peristome; *Amp.* ampulla, probably a deeply staining enlargement in the ectoplasmic thickening; *An.M.* anterior membrane of karyophore; *Bas.P.* basal plate; *C.V.* contractile vacuole; *C.V.C.* contractile vacuole canal; *Cyt.T.* cytopharyngeal tube or continuation tube; *Cytoph.* cytopharynx; *Cytopyge*, cytopyge; *Ect.* ectoplasmic thickening along the floor of the cytopharynx; *Mic.* micronucleus; *Pos.M.* posterior membrane of karyophore.

Fig. III. Cysts of *Nyctotherus uichancoi* drawn from life. Approx. ×511. A, mature cyst with macronucleus, surrounded by glycogen-like platelets, at narrow end away from operculum. B, excysting form. The ciliary apparatus is re-formed and the organism has reversed its position in the cyst. The inner surface of the operculum is corroded. C, ciliate making its exit from the cyst.

appears to be a continuation of the suture fibre. This longitudinal fibre appears to be single and lies just inside the basal plates. It is undoubtedly homologous with the "longitudinale Fibrille der Präoralhöhle" of *Nyctotherus ovalis* and *N. cordiformis* as described by Ten Kate (1927).

The membranelles lie with their basal plates at right angles to the peristome. The largest are those in the cytostomal region. Their size decreases anteriorly

up the peristome and posteriorly down the cytopharynx. The action of the membranelles is not the same at different levels of the peristome-cytopharynx. Those of the peristome and mouth region beat intermittently and are the first to cease their activity as the ciliate becomes quiet, while those of the cytopharynx beat steadily and continue to do so even after the peripheral cilia have ceased to beat.

Each membranelle is composed of three rows of cilia, originating in the basal plate and fusing distally to form a flat paint-brush-like element. This is at variance with the observation of Maier (1903) on *N. cordiformis*. He described and figured only two rows in this species. My observations are in accord with those of Lund (1935) on the membranelles of *Oxytricha*. I have never been able to differentiate the individual basal bodies in the deeply staining plate, if indeed they exist as units. McDonald (1922) was of the opinion that the basal bodies may be fused in the basal plates of *Balantidium* as was also believed by Bishop (1927) in *Spirostomum ambiguum*, although earlier authors (Johnson, 1893; Maier, 1903; Schröder, 1906) had described the individual basal bodies in the basal plates of *Stentor* and other heterotrichous ciliates.

Many observations on sectioned material have led me to the conclusion that the cytopharyngeal region of *Nyctotherus uichancoi* is of much simpler construction than those of *N. ovalis* (Ten Kate, 1927; de Mello *et al.* 1934), *N. piscicola* (Entz, 1913), *N. cordiformis* (Ten Kate, 1927) or *N. silvestrianus* (Kirby, 1932). I have prepared and examined sections of *N. ovalis* from *Periplaneta americana* and Dr Kirby has sent me preparations of *Nyctotherus silvestrianus* for comparison. *N. uichancoi* appears to lack a great many of the accessory fibres and elements found in the above-mentioned species.

The cytopharynx of *N. uichancoi* appears to be made up of an invagination of the ectoplasm of which the walls are very thick, homogeneous, and faintly staining with haematoxylin (Fig. II B). The ectoplasmic thickening along the floor of the cytopharynx is very noticeable in sections as well as in whole mounts (Fig. II A and B, *ect.*), and in material insufficiently destained may be black. I have never found anything to correspond with the "subpharyngeal canal" of Higgins (1929), although a bulge in the ectoplasmic wall probably represents the "ampulla" of *N. cordiformis* (Fig. II A, *amp.*). This bulge retains the stain longer than the rest of the cytopharyngeal wall, as in *N. cordiformis* according to Higgins. I cannot say whether or not this structure possesses any specialized function in *N. uichancoi*.

The contractile vacuole is located near the posterior end of the body toward the right margin. It is formed by fusion of a number of small vacuoles in the endoplasm of that region. When ready to discharge it measures about 17 μ in diameter in large individuals. At systole the vacuole comes in contact with the inner end of a permanent canal through which its contents are emptied (Fig. II A, *c.v.c.*; Figs. 21–23). This canal is always plainly visible in life as well as in fixed material. It is formed from a continuation of the cytopyge. The

cytopyge is large and is an indentation of the posterior ectoplasm. There is some variation in its shape and size from a slit to an oval concavity. The latter condition is most frequently encountered. I have never observed defaecation of solid material through this opening (as recorded by Higgins (1929) in *N. cordiformis*), but it is possible that it may function in that capacity as well as being an outlet for the contractile vacuole.

The most distinctive feature of *N. uichancoi* is the macronucleus, which is clearly visible in life. In adult individuals the shape is constant, while in small newly excysted ciliates it is spherical, as would be expected (Fig. 23).

The adult macronucleus, when viewed from the ventral surface, is triangular and slightly arched along its anterior side. The posterior portion extends beyond the level of the cytopharynx and ventral to it (Fig. 22). In dorsal view at a high focus it appears spindle-shaped with its long axis parallel to the lateral body axis (Fig. II A; Fig. 21). When viewed from the lateral edge of the body it is seen to be in the form of a weak hook, the handle of which is directed posteriorly, ventral to the cytopharynx, while the bent portion turns in a dorsal direction, anterior to the cytopharynx (Fig. 11 B). This shape is relatively constant and can be detected at once in the living ciliate, although fixation in sublimate-acetic causes the macronucleus to contrast somewhat, minimizing the characteristic shape.

The chromatin of the adult macronucleus is concentrated into homogeneous spheres which are uniformly distributed. They stain intensely with haematoxylin and the Feulgen reagent and can be seen in life as refrigent spherules. In small newly excysted individuals these spheres may reach three times the volume of the adult condition (Fig. 23).

The micronucleus is comparatively large (4–5 μ) and is easily visible at all times. It is situated close to the anterior-dorsal portion of the macronucleus. It is composed of homogeneous deeply staining chromatin and is surrounded by a conspicuous membrane. It can easily be seen in life. There is very little variation in the position of the micronucleus in adult trophozoites, except during fission.

Surrounding the macronucleus is a conspicuous karyophore. It is made up of two membranous sheets which appear completely to separate the anterior and the posterior endoplasms. They appear to be continuations of the ectoplasmic layer and are attached at all sides. This attachment can be seen in lateral view as well as the conventional dorsal or ventral views. The anterior sheet (Fig. II, *an.m.*) covers the entire macronucleus anteriorly and may be attached to the micronucleus as well. The posterior sheet (Fig. II, *pos.m.*) is irregular and conforms to the shape of the posterior portion of the macronucleus.

From my observations I cannot agree with Grassé (1928) that the karyophore of *Nyctotherus* is fibrillar. I am more inclined to the belief of Zulueta (1916) that it forms a septum, at least in *N. uichancoi*. This double-layered septum only exists in the resting trophozoites, however, and it completely

disintegrates during fission and within the cyst. Newly excysted individuals possess no karyophore.

What appears to be a frontal lamina (as in *N. haranti* and *N. gyoeryanus* Grassé, 1928, and *N. silvestrianus* Kirby, 1932) has been seen in only one specimen of *N. uichancoi*. This was from *Panesthia spadica*. In all of the hundreds of individuals examined from *P. javanica* the anterior and the posterior membranes appear to be attached at about the same level, with no indication of a frontal lamina.

The posterior endoplasm of *Nyctotherus uichancoi* is granular and contains many food vacuoles and small water vacuoles. The food vacuoles contain minute inclusions, probably bacteria. I have never found any evidence that this ciliate ingests fragments of wood. Aggregates of bacteria-like bodies are often found lying free in the endoplasm, but these are probably similar to the parasitic bacteria described by Sassuchin (1928) in *N. ovalis* and by Kirby (1932) in *N. silvestrianus*.

The endoplasm anterior to the macronucleus is finely granular, and in large trophozoites is packed with ovoid refractile bodies (Figs. 21–22). These correspond in all respects to the glycogen platelets of Grassé (1928). They do not stain with haematoxylin but stain brown with iodine. These bodies are numerous in ripe cysts but are scarce in old cysts, especially when protoplasmic activity is resumed before excystation. In small newly excysted individuals they are absent (Fig. 23).

Typical *Nyctotherus* cysts are plentiful in the hind gut of *Panesthia javanica* but I have not found them as yet in *P. spadica*. They resemble those of *Nyctotherus ovalis* (Lucas, 1927, 1928), being narrow at one end and possessing an operculum at the more truncated end. The majority measure from 55 to 90 μ in length, but occasionally smaller ones (25 μ) are found. These smaller cysts (Fig. VIII H) are usually more rotund than the larger ones. It is undoubtedly from these that the minute ciliates, found less frequently than the medium-sized to large forms, emerge.

The cysts are thick-walled structures, yellowish in life. Their contents stain deeply with haematoxylin, while with the Feulgen reagent the macronucleus and micronucleus show clearly. In sectioned material fixed in Zenker's fluid and stained with Mallory's triple stain the cyst wall stains a brilliant orange, the cytoplasm blue, both nuclei bright red, and the glycogen platelets remain clear. No evidence of any fibrillar or ciliary organization can be seen in a mature cyst. In life a large vacuole is usually present near the operculum (Fig. III A). This vacuole starts pulsating as the ciliate emerges from the cyst.

I have watched the process of excystation from the time protoplasmic activity began until the young ciliate had emerged. In general my observations agree with those of Lucas (1928) for *N. ovalis*, but there are a few points of disagreement which I will consider briefly.

In the ripe cyst of *N. uichancoi* the disposition of the nuclei and glycogen platelets are as in *N. ovalis*. Lucas observed body striations and other evidence

of the old organization which are completely lacking in the mature cyst of *N. uichancoi*. The first evidence of excystation is a slight cyclosis. Soon afterwards the peripheral cilia are reformed and begin beating actively. During the reorganization of the peripheral cilia the cytopharynx can be detected as a light band near the macronucleus. Gradually the membranelles make themselves evident. By this time the ciliate is spinning and twisting within the cyst. Eventually its movements cause it to reverse its position within the cyst wall, so that finally the end possessing the macronucleus is lying nearest the operculum. Lucas was of the opinion that there was a reversal of polarity in *N. ovalis*, the nuclei migrating through the cytoplasm towards the operculum. This is certainly not the case in *N. uichancoi*.

Apparently through the action of a digestive ferment the inner surface of the operculum becomes pitted, and gets thinner and thinner (Fig. III B). Finally the operculum seems to dissolve leaving a pore. Sometimes projections, probably the sides of the operculum, can be seen attached to the remaining cyst wall. Through the pore, the active elastic ciliate forces its way (Fig. III C). All the adult structures are present by this time, except the karyophore and the glycogen platelets. The macronucleus is spherical, as it was in the cyst.

Diagnosis: Length of adult 106μ (78–145μ), width 77μ (60–90μ). Length of small excysted forms 40μ (26–92μ), width 23μ (15–62μ). Cysts 71μ (25–90μ) shaped like the cysts of *N. ovalis*. Cytopharynx long and recurved spirally, ending to the right of the midline towards the ventral surface. Macronucleus hook-shaped in lateral view, partially surrounding the cytopharynx. Micronucleus large, situated dorsal and anterior to the macronucleus. Karyophore present. Glycogen-like bodies abundant anterior to the nuclei. Bacteria feeder.

Type host: Panesthia javanica from Mt. Maquiling, Luzon, Philippine Islands.

Additional host: Panesthia spadica from Gifu, Japan.

General features of the family Clevelandiidae fam.nov.

This heterotrichous family is represented by 9 species belonging to 2 genera. It is characterized as a whole by the posterior position of the cytostome and the anteriorly directed cytopharynx. The various species range in length from 16 to 206μ (Fig. IV). These ciliates structurally resemble *Nyctotherus* closely.

Orientation.

If we were to follow the dictates of some workers (see de Morgan, 1925) it would be necessary to regard the oral end as anterior in all cases. This would necessitate the disregarding of all other structures, however, and also the direction of motion. It would thus be difficult to account for the transition from a *Nyctotherus* to the type under consideration. So before describing the family Clevelandiidae I will give the nomenclature to be used here regarding

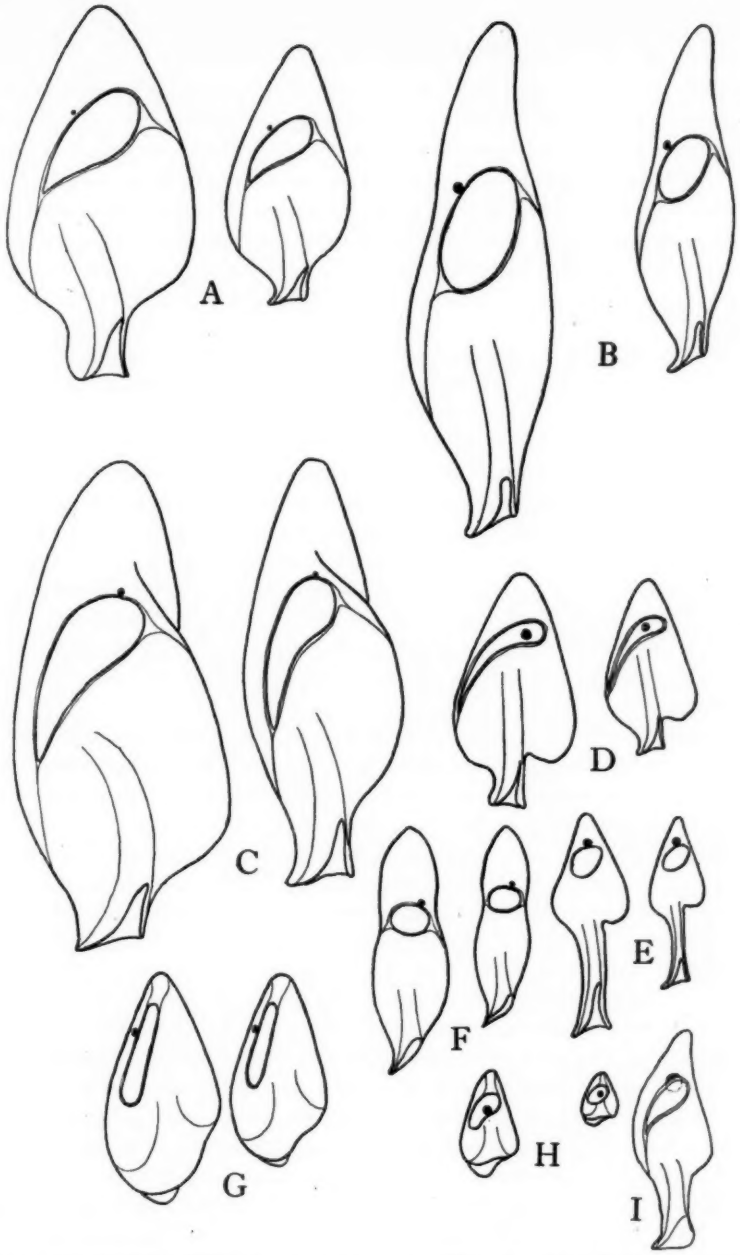

Fig. IV. Camera lucida drawings of members of the family Clevelandiidae to illustrate comparative sizes. In all but one case (*I*) the largest and the smallest of each species are shown. All material fixed in Bouin's fluid except *I*, which was fixed in Schaudinn's fluid. ×400. A, *Clevelandia panesthiae*. B, *C. elongata*. C, *C. contorta*. D, *C. parapanesthiae*. E, *C. hastula*. F, *C. constricta*. G, *Paraclevelandia simplex*. H, *P. brevis*. I, *Clevelandia nipponensis*.

orientation. I shall regard the end at which the macronucleus is found as anterior, as in *Nyctotherus*; the end bearing the cytostome, contractile vacuole, cytopyge, and posterior suture, as posterior; the surface bearing the notch in the peristomal projection as ventral; and the margin nearest to which the contractile vacuole lies as left. By employing these terms in this sense it is possible to compare the structures found in the members of this family directly with those of the genus *Nyctotherus*.

External characters.

The members of this family vary considerably in outline. They all tend to be pointed anteriorly and to be truncated obliquely at the posterior oral end. Distinct dorso-ventral flattening occurs in all species of both genera but is slightly more pronounced in *Paraclevelandia* than in *Clevelandia*. The body may be relatively elastic, as in *Clevelandia elongata*, *C. constricta* and *C. hastula*; it may be rigid, as in *Paraclevelandia brevis* and *P. simplex*; or it may be only slightly elastic, as in the remaining species. Contractility is entirely absent. *Clevelandia constricta* and *Paraclevelandia simplex* are nearly bilaterally symmetrical but in the remaining species there is a distinct tendency toward curvature, especially at the anterior end. This curvature is towards the left. Spiral torsion of the body is seen in one species only, *Clevelandia contorta*, where a single leiotropic twist forms a deep groove in the left margin of the body.

The pellicle is thick, varying from that of the genus *Nyctotherus* to the heavy refractile coat of *Paraclevelandia simplex*. It is deeply striated in a longitudinal direction anteriorly but the striations curve into a whorl just under the contractile vacuole.

The peripheral rows of cilia are placed in the depressions between the pellicular folds and follow them throughout their length. Each row is composed of a longitudinal co-ordinating fibre connecting the numerous basal bodies. Most of the ciliary rows originate in a suture on the right margin of the body which may extend to the level of the contractile vacuole, as in *Clevelandia hastula*, *C. nipponensis*, *Paraclevelandia brevis*, and *P. simplex*, or it may be limited to the anterior third of the body, as in *Clevelandia elongata* and *C. panesthiae*. The rows proceed dorsally and ventrally around the body and end in a distinct whorl just ventral to the contractile vacuole and very near the cytopyge. As the ciliary rows approach the whorl from all directions they unite so that the whorl itself is a network. The ciliary rows of the oral projection and those just anterior to it seem to be continuous loops, starting from one side of the whorl, looping around the body, and returning to the opposite side. This whorl is undoubtedly homologous with the posterior suture of *Nyctotherus uichancoi*.

The number of ciliary rows varies greatly among the different species. In some they are far apart (as in *Clevelandia hastula* and *C. nipponensis*) while in others they are closely set.

The ciliary action is similar in all species. The cilia beat metachronously and rather slowly so that distinct waves can be seen passing backwards to the whorl. Upon nearing the whorl the waves break, the cilia beating in all directions. None of the members of this family moves as rapidly as *Nyctotherus uichancoi*. They swim through the intestinal debris with a steady forward motion, pausing often to alter their direction. In the species possessing a long oral projection (*Clevelandia nipponensis*, *C. hastula* and *C. parapanesthiae*) the movement is usually somewhat jerky owing to the thrashing of this structure. *C. elongata* bends during swimming more than do the other species. This bending occurs just below the level of the contractile vacuole, and the anterior portion of the body may bend as much as 90 degrees.

Internal characters.

Oral apparatus. The cytostome lies at the base of a funnel-shaped peristome, and is large in all species. The peristomal membranelles are very long and lie among the right posterior side. They are placed with their basal plates perpendicular to the long axis of the body in a plane corresponding to the dorsoventral body axis (Fig. VI, *bas.p.*). The membranelles follow the margin of the invaginated peristomal funnel and continue into the cytopharynx.

The cytopharynx is usually nearly uniform in diameter and leads anteriorly from the mouth into the body. Slight to decided curvature occurs toward the right, and in some species the cytopharynx dips ventrally at its proximal end. Its walls are rather thick and, as in *Nyctotherus uichancoi*, are composed of a homogeneous substance continuous with the ectoplasm. The right wall is thicker than the left, while the latter is thicker than the dorsal or ventral. In *Clevelandia constricta* a "continuation tube", similar to that of *Nyctotherus*, may occur. Its presence, length, and direction are highly variable.

The membranelles are smaller and less pointed towards the distal end of the cytopharynx than towards the mouth. They beat inwardly so that a current is created which tends to draw floating particles into the cytostome and cytopharynx.

Contractile vacuole. Except for size the contractile vacuoles of all species of this family are similar. They all originate in the same fashion, occupy the same relative position in the body, and all discharge their contents through a permanent slit-like cytopyge.

In the endoplasm to the left of the cytopharynx a number of small vacuoles slowly but continuously form. These fuse to form the large contractile vacuole. It is noticeably sluggish in its pulsation in all species, so that the majority of fixed specimens show this organelle. The position in the various species can be seen in the figures.

Just ventral to the contractile vacuole and near the whorl of peripheral cilia occurs a slit-like cytopyge. Through this permanent slit the contractile vacuole empties its contents. No canal can be detected, but the contractile vacuole comes in contact with the interior edge of the cytopyge just before

systole. In most species the cytopyge is not conspicuous, but in some, notably *Clevelandia constricta*, the edges may protrude from the margin of the body (Fig. 39).

Nuclei. The macronucleus varies in shape and size among the different species of this family. In 4 species it is in the form of a thick crescent, truncate at the anterior end and pointed posteriorly. The long axis of the crescent is oblique from left to right. In 4 species the macronucleus is ovoid to spherical, while in the genus *Paraclevelandia* it is nearly cylindrical with its long axis parallel to the long axis of the body.

The chromatin is disposed in spheres, of varying sizes, which stain intensely with all nuclear dyes and fill the macronucleus.

The micronucleus is a spherical or ovoid body, made up of a nuclear membrane and dense chromatin, varying in size and staining capacity among the different species. In most cases it is easily seen even in living organisms. It may lie ventral, dorsal, or lateral to the macronucleus, or it may lie just anterior to it. In some species (*Clevelandia nipponensis*, *C. parapanesthiae* and *Paraclevelandia brevis*) the micronucleus stains so intensely with haematoxylin that it remains black after the macronuclear chromatin is completely destained. This intensity is seen also after the Feulgen reaction. In some other species the micro- and macronuclear chromatin stain equally, while in *Clevelandia panesthiae* and *Paraclevelandia simplex* the micronuclear chromatin stains less intensely than that of the macronucleus.

Karyophore. Like members of the genus *Nyctotherus* s.str. of Grassé (1928) most of the members of this family possess a nuclear suspensory apparatus in the form of a karyophore. In some of the species it is made up of two complete membranes as in *N. uichancoi*, in others of one membrane only which loops around the macronucleus from the right. In one species (*Clevelandia hastula*) I have been unable to identify with certainty any membrane about the nucleus. In both species of *Paraclevelandia* the karyophore is peculiar in that it originates from the anterior ectoplasm near the lateral walls of the sac-like structure (to be described later) and forms a bag in which the macronucleus hangs.

The occurrence of a frontal lamina seems to be limited to one species, *Clevelandia constricta*, and may be observed best in very lightly stained preparations. I have seen this elongation of the anterior membrane of the karyophore in a number of individuals and it may occur in all. It appears to stretch entirely across the anterior tip of the body (Fig. 39) and forms the boundary line between a heavily granular region and the less granular area anterior to the macronucleus.

Inclusions. In the ectoplasmic layer of *Paraclevelandia simplex* are numerous refringent bodies. They are irregular in shape and are lined up between the rows of basal bodies. They do not stain after any of the fixatives employed but form a highly characteristic vacuolated-appearing coating over the endoplasm. They may be vacuoles, but their irregular shape makes this

assumption doubtful. Since this species does not possess the typical endoplasmic glycogen-like platelets it may be that the stored food material is represented by these ectoplasmic bodies. If this is so it is strange that they are absent from *P. brevis*, which also lacks endoplasmic glycogen-like platelets.

All species of *Clevelandia* possess typical glycogen-like platelets, similar to those of *Nyctotherus*. They are usually confined to the endoplasm anterior to the macronucleus, but occasionally may be found scattered in the posterior endoplasm as well. These cases, found infrequently in all the species of *Clevelandia*, probably represent newly divided forms in which the karyophore of the parent, disintegrating for division, has allowed the general distribution of the platelets. The platelets appear similar in every respect to those found in *Nyctotherus uichancoi*.

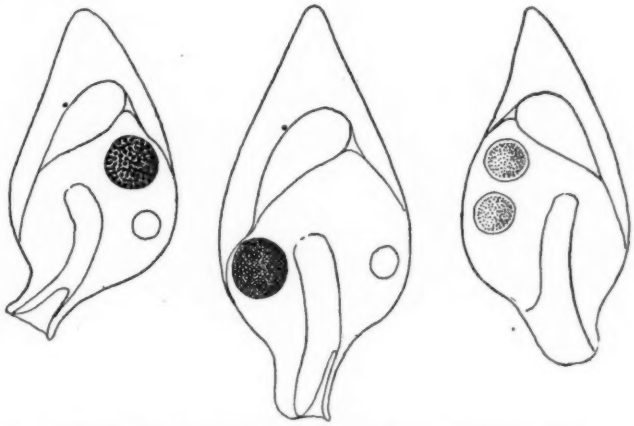

Fig. V. *Clevelandia panesthiae* showing various stages of development of the parasitic organism *Sphaerita*. Bouin's fluid-Delafield's haem. ×500.

Food inclusions of what appear to be dense masses of bacteria occur in the posterior endoplasm of all species. No wood has been observed in the food vacuoles. The food inclusions are always limited to the region posterior to the karyophore in those species possessing two membranes, but occasional food inclusions may be carried around the macronucleus in those species possessing an incomplete karyophore.

In addition to the food inclusions and the glycogen-like platelets most, if not all, of the species are parasitized by rod-shaped or spherical bacteria-like organisms. These usually occur in clusters and may be particularly abundant to the left of the cytopharynx. In *Clevelandia panesthiae* parasitism by the micro-organism *Sphaerita* is common (Fig. V), but I have not encountered it in any of the other ciliate species in *Panesthia*. The incidence of parasitism is very high in some roaches, as many as 50 per cent of *Clevelandia panesthiae* being infected, though in others the parasites are absent.

Neuromotor system. The fibrillar systems of the various species of this family are so nearly identical that they may be treated as one. Indeed, the only differences that I have been able to note, from a careful study of sectioned material and whole mounts, are in the number and extent of some of the peristomal and cytopharyngeal radiating or cross fibres. These differences are correlated with the number of membranelles present. A study of the fibrillar system emphasizes the compactness of this family as a taxonomic group.

The membranelles are like those of *Nyctotherus uichancoi* in structure. Each one is made up of three rows of fused cilia (Fig. VII, *memb.*) originating in a

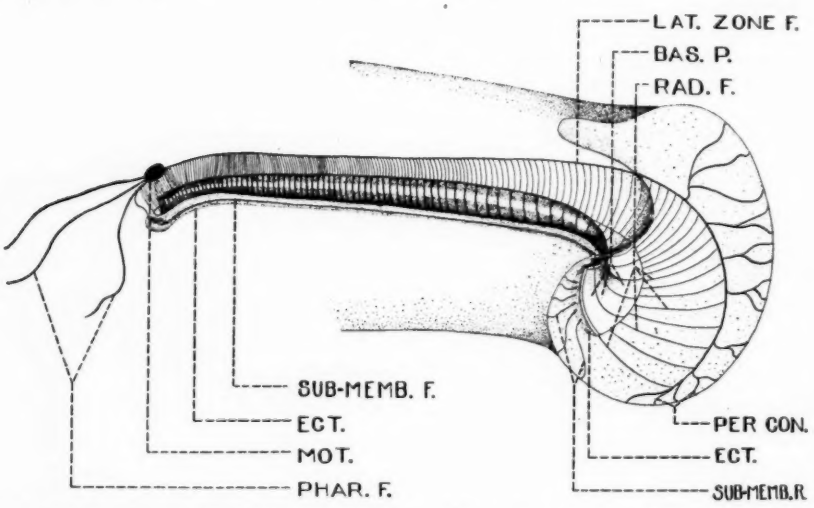

Fig. VI. Diagram illustrating the fibrillar system of the oral region of members of the family Clevelandiidae, based on *Clevelandia elongata*. A ventral view. Abbreviations: *Bas.p.* basal plates; *Ect.* ectoplasmic thickening upon which basal plate rests. *Lat.zone f.* lateral zone fibre; *Mot.* motor mass or motorium; *Per.con.* peristomal connectives; *Phar.f.* pharyngeal fibres running into the endoplasm from the motorium; *Rad.f.* radiating fibres connecting the lateral zone fibre with the ends of the basal plates. *Sub-memb.f.* submembranellar fibres; *Sub-memb.r.* roots of the submembranellar fibres.

bar-like basal plate. I have never been able to see any indication of the individual basal bodies of the cilia in this plate. It stains very intensely with haematoxylin and with the acid fuchsin of the Mallory triple stain. The rows of basal plates lie on the ectoplasmic thickening of the peristomal and cytopharyngeal wall towards the lumen (Figs. VI and VII, *ect.*). Along the inner edge of the ectoplasmic thickening is a set of longitudinal fibres, usually 6 in number, that extend from the distal end of the cytopharynx along its length and out through the wall of the peristome (Figs. VI and VII, *sub-memb.f.*). Here they become thinner and flare out (Fig. VI, *sub-memb.r.*) to loop around the lip of the peristome. Whether or not they join any of the co-ordinating

fibres of the peripheral system could not be determined. It seems probable that this is so. In cross-sections through the cytopharynx the basal plates, ectoplasmic thickening, and the submembranelle fibres can be seen clearly (Figs. 31–32). I have never been able to detect any fibrillar connexions through the ectoplasmic thickening between the 6 submembranelle fibres and the plates. If impulses are transmitted directly to the basal plates from these fibres they must pass through the substance of the ectoplasmic thickening.

The submembranelle fibres unite at the distal end of the cytopharynx and fuse with a conspicuous body on its left border. This body is usually ovoid,

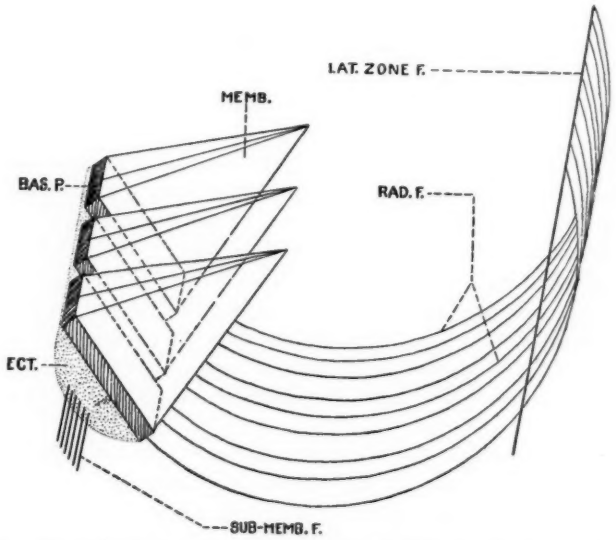

Fig. VII. Diagram showing the relationships of the fibres of the oral region and the membranelles. Abbreviations as in Fig. V.

refractile in life and deeply staining with haematoxylin and the acid fuchsin of Mallory's triple stain. It occupies the same relative position as the ampulla of *Nyctotherus uichancoi* (described above) and may be homologous with that structure. However, it can clearly be differentiated as a structure apart from the ectoplasmic wall of the cytopharynx, though this is not possible in *Nyctotherus*. As all of the fibres of the peristome-cytopharynx complex are either directly or indirectly connected to this structure, and it obviously resembles the motoria of other ciliates, I shall call it the motorium (Fig. VI, *mot.*). Unlike some of the motoria of other ciliates those occurring in the family Clevelandiidae are always visible, even in whole mounts, as a glance at the figures will show (Pls. II and III). Also sectioned material shows that the criticism of Rees (1931) does not apply to these forms.

A varying number of fibres leaves the motorium, extending into the

cytoplasm in various directions (Fig. VI, *phar.f.*). These are unquestionably homologous with the pharyngeal strands of such forms as *Boveria teredinidi* (Pickard, 1927), *Glaucoma* (*Dallasia*) *frontata* (Calkins & Bowling, 1929), *Eupoterion pernix* (MacLennan & Connell, 1931), *Morgania* (*Conchophthirius*) *mytili* (Kidder, 1933) and *Metopus circumlabens* (Lucas, 1934). They appear to end blindly in the endoplasm posterior to the macronucleus.

A single rod-like fibre runs from the motorium posteriorly along either the left or the ventral border of the cytopharynx. It (Figs. VI and VII, *lat.zone f.*) follows the lip of the peristome curving over to the right where it becomes thin and appears to loop around the lip, in much the same manner as the submembranelle roots. Along its curve, in the dorsal wall of the peristome, it gives off numerous fine fibres that also loop around the peristomal lip and are lost from view (Fig. VI, *per.con.*). In some species the curve of the lateral zone fibre is so close to the edge of the peristomal lip that no connexions can be seen.

Every basal plate is connected to the lateral zone fibre by 3 fine cross or radiating fibres. These originate from the dorsal or inner ends of the plates and follow the curvature of the peristomal and cytopharyngeal walls to fuse with the lateral zone fibre (Figs. VI and VII, *rad.f.*; Figs. 26, 31, 32 and 38). In the peristomal region they can plainly be seen in life as well as in whole mounts. I have traced this set of fibres in longitudinal and cross sections in every species of the family Clevelandiidae with the exception of *Clevelandia nipponensis*. Of this species I have had no material for sectioning, but most of the fibres can be seen in the whole mounts and they correspond with those of *C. hastula*.

It can be seen that the above system of fibres corresponds with the classical neuromotor systems in ciliates. Most of the previous work, however, has been done on holotrichous, oligotrichous, and hypotrichous forms. Therefore it is interesting to find a group of heterotriches that possess a compact system of easily observable fibres which are comparable in every respect with the well-known forms. From the above description it is clear that, on morphological grounds, there is reason to believe that at least the oral ciliation may be controlled by the motorium. If the peristomal connectives and the submembranelle roots connect in some way with the peripheral system, as I believe probable, then we have a well integrated system of fibres controlling the activity of all the motor organelles.

Occurrence of ciliate genera and species in Panesthia javanica *and* Panesthia spadica.

Panesthia javanica was found to be much more heavily infected with ciliates than was *P. spadica*. Also more species occurred in the former host (9 in *P. javanica*; 7 in *P. spadica*). In many individual roaches not all of the normal species were found together, although this frequently occurred. Also there was some variation as to prevalence of certain species in different

Table I

Ciliates	*P. javanica* I	II	III	IV	V	VI	VII	VIII	IX	X	*P. spadica* I	II	III	IV	Number of roaches infected: *P. javanica*	*P. spadica*	Total per species: *P. javanica*	*P. spadica*
Nyctotherus uichancoi	7	3	.	43	19	1	5	15	6	2	2	.	.	.	9	1	101	2
Clevelandia panesthiae	48	4	1	1	26	7	45	45	35	9	15	.	.	.	10	1	221	15
elongata	1	.	.	.	3	.	19	20	1	2	.	.	.	.	6	.	46	.
contorta	.	.	.	.	.	.	2	3	.	8	17	.	.	.	3	1	13	17
parapanesthiae	6	7	5	.	7	6	3	1	7	9	.	.	.	.	9	.	51	.
hastula	11	3	2	.	1	6	2	4	.	5	.	.	.	.	8	.	34	.
nipponensis	.	.	.	.	.	.	.	.	.	.	7	.	.	.	.	1	.	7
constricta	.	15	6	7	.	.	.	.	5	6	1	2	1	.	5	3	39	4
Paraclevelandia brevis	99	23	24	3	3	18	79	80	3	4	15	3	.	9	10	3	336	27
simplex	.	.	1	.	.	.	9	10	19	1	1	.	1	.	5	2	40	2
Total per sample	172	55	39	54	59	38	164	178	76	46	58	5	2	9		Total	881	74

individual hosts. In order to present an estimate of the conditions, sample counts were made.

All the species of ciliates present on a single slide from each of 10 *P. javanica* and 4 *P. spadica* were counted, and the species recorded separately, the method being a modification of that employed by Kofoid & MacLennan (1930) in their studies on the ciliates from *Bos indicus*. The results, summarized in the table, give an estimate of the incidence of infection, the occurrence of the various ciliate species, and the intensity of infection of the two hosts. It should be noted, however, that one slide represents only a small sample from each roach and the actual figures should be multiplied many times to record all the individual ciliates from a given insect.

Systematics of the family Clevelandiidae fam.nov.

This family of ciliates obviously falls into the subclass Spirotricha and the order Heterotrichida (classification of Calkins, 1933). It is also obviously related most closely to the family Plagiotomidae, to which the genus *Nyctotherus* belongs.

CLEVELANDIIDAE fam.nov.

Diagnosis: Heterotriche ciliates, elongate, ovoid or spear-shaped with the mouth at the posterior end. Body completely ciliated. Peristome and cytopharynx bearing membranelles from the basal plates of which radiating fibres arise. Usually 1 macronucleus and 1 micronucleus. Karyophore usually present. A single contractile vacuole near the posterior end on the left side, opening to the outside through a slit-like cytopyge.

Type Genus: Clevelandia from *Panesthia javanica* and *P. spadica.*

Clevelandia gen.nov.

Diagnosis: More or less flexible Clevelandiidae with peristome at the end of an elongated projection from the posterior end of the body. Body elongate or spear-shaped. Glycogen-like platelets present in the endoplasm. All known species occur in the hindgut of wood-feeding roaches.

Type Species: Clevelandia panesthiae from *Panesthia javanica* and *P. spadica.*

Clevelandia panesthiae sp.nov. (Figs. 24–25).

Diagnosis: Average length 123 μ (87–156 μ), average width 62 μ (53–78 μ). Macronucleus tear-drop-shaped with point toward the right margin, average length 42 μ. Micronucleus small (2–3·5 μ) situated just anterior to macronucleus and slightly dorsal. Distinct karyophore present consisting of 2 membranes. Left body margin curved more or less evenly towards peristomal projection. Peristomal projection relatively short.

Description: The body is slightly flattened dorsoventrally. The anterior end is usually pointed, although in some individuals less than in others. The right body margin is usually slightly concave towards the anterior end but convex in the posterior two-thirds. The left body margin is evenly convex until just

below the level of the contractile vacuole where it dips sharply toward the peristomal projection. The peristomal projection is not over one-fifth the entire body length, and occurs near the mid-line of the body. The peristome is nearly enclosed, there being only a V-shaped slit opening laterally on the ventral border towards the left. The peristomal connective or radiating fibres line about one-half the circumference of the peristome and cytopharynx. The macronucleus is thick and slightly curved and lies with its long axis in an oblique direction from the anterior left to the posterior right. The micronucleus is inconspicuous owing to its small size and lack of affinity for nuclear dyes. The karyophore is made up of 2 thick membranes that originate from the ectoplasm posterior to the macronucleus, about mid-region on the left and very near the peristomal projection on the right. It sharply separates the endoplasm posterior to the macronucleus from the rest of the body. In this posterior region lies the opening of the cytopharynx, and the endoplasm is conspicuous by its numerous food inclusions. Parasitism by *Sphaerita* is common.

Occurrence: Clevelandia panesthiae occurs regularly in the hindgut of all individuals, large and small, of *Panesthia javanica*, and in most individuals of *P. spadica*. It is one of the commonest species encountered in these hosts.

Type host: Panesthia javanica from Mt. Maquiling, Luzon, Philippine Islands.

Additional host: Panesthia spadica from Gifu, Japan.

Clevelandia contorta sp.nov. (Figs. 27–28).

Diagnosis: Average length 179 μ (160–185 μ), average width 80 μ (71–92 μ). Macronucleus shaped as in *Clevelandia panesthiae*. Micronucleus small and situated near anterior border of the macronucleus. Karyophore as in *C. panesthiae*. Body twisted leiotropically resulting in a deep spiral groove which is most apparent on the left margin of the body.

Description: This species is very conspicuous because of the twisted condition of the body. In life the twist is more noticeable than in fixed material because of the resulting characteristic spiral swimming motion. In its general organization it resembles *C. panesthiae* except that the peristomal projection is displaced to the right of the mid-line and the body size is always greater. The organization of the cytopharynx is similar to that of *C. panesthiae*. Although the karyophore is as complete as that of *C. panesthiae* there is a less noticeable food area.

Occurrence: This is the rarest species of ciliate in *Panesthia*. It never occurs abundantly and the incidence of infection is low. From the material available it would seem that the incidence is slightly higher in *P. spadica* than in *P. javanica* (see table on p. 189).

Type host: Panesthia javanica from Mt. Maquiling, Luzon, Philippine Islands.

Additional host: Panesthia spadica from Gifu, Japan.

Clevelandia elongata sp.nov. (Figs. 29–32).

Diagnosis: Body long and worm-like, slightly flattened dorsoventrally. Anterior end pointed and twisted to the left. Average length 188 μ (143–206 μ), average width 69 μ (57–81 μ). Macronucleus ovoid lying in the mid-region of the body with its long axis oblique to the long axis of the body. Micronucleus large (6–8 μ) lying dorsal to the macronucleus and towards its anterior end. Peristomal projection variable in length from one-ninth to one-seventh of the entire body length. Body capable of bending, especially in the posterior portion.

Description: This is the longest species of ciliate encountered from *Panesthia*. The general body shape and the worm-like swimming and creeping motions make it easily distinguishable in life. Owing to the accentuated bend at the anterior end it appears asymmetrical. In some individuals the peristomal projection is much longer than in others, but in all cases the cytopharynx is approximately the same length. I have never seen any evidence of contractility and I am convinced that the differences in length of this structure are due to individual variation. The radiating fibres line less than half of the circumference of the cytopharynx, and there is a conspicuous region between the curved lateral zone fibre and the edge of the peristomal lip. This region bears the peristomal connectives (see Fig. VI). The macronucleus is relatively large and ovoid and is never pointed at its posterior end, as in *Clevelandia panesthiae*. The micronucleus stains very deeply and is easily seen in all preparations. The karyophore is of the same type as that of *C. panesthiae* but is more delicate. Food inclusions are usually few in number. On the surface of the body the ciliary rows are more numerous and more closely set than in any of the other species in *Panesthia*.

Occurrence: Clevelandia elongata is common in *Panesthia javanica* but has not been found in *P. spadica*. It sometimes occurs in considerable numbers and because of its large size is one of the first species noticed in an intestinal examination.

Type host: Panesthia javanica from Mt. Maquiling, Luzon, Philippine Islands.

Clevelandia constricta sp.nov. (Figs. 37–39).

Diagnosis: Body elongate and nearly cylindrical, being only slightly flattened dorsoventrally. Anterior end bluntly pointed. Body constricted conspicuously to slightly in the region of the macronucleus. Average length 84 μ (65–97 μ), average width 30 μ (22–37 μ). Macronucleus evenly ovoid, situated just anterior to the mid-region with its long axis parallel to the transverse axis of the body. Micronucleus anterior and to the left of the macronucleus. Karyophore present, consisting of 2 membranes. Peristomal projection short.

Description: This is the most symmetrical member of the genus. In some cases slight torsion occurs in the anterior third of the body but the majority

are straight. The macronucleus is always evenly ovoid and the karyophore membranes appear to attach slightly below its level. The micronucleus stains rather faintly but is easily visible. The cytopyge is very conspicuous. The cytopharynx is straight and the radiating fibres extend around three-fourths of its circumference. Around the macronucleus, anterior to the karyophore, is a zone of what appear to be parasitic bacteria. They are characteristic of this species as to localization. In addition to these there are clusters of micro-organisms in the posterior endoplasm. Food bodies are scarce.

Occurrence: Clevelandia constricta is found in the majority of *Panesthia*. In some cases they are abundant while in others their number is small. They alone, of all the species of ciliates from these hosts, are concentrated in the extreme posterior end of the hindgut. A few of the other species are also regularly present in this region and a few *Clevelandica constricta* are found throughout the hindgut, so the localization is not absolute.

Type host: Panesthia javanica from Mt. Maquiling, Luzon, Philippine Islands.

Additional host: Panesthia spadica from Gifu, Japan.

Clevelandia nipponensis sp.nov. (Figs. 33–34).

Diagnosis: Body spear-shaped, sharply pointed anteriorly, slightly flattened dorsoventrally, mid-region nearly cylindrical, bulging on the left margin at the level of the contractile vacuole. Peristomal projection slightly less than one-third the entire body length. Cytopharynx one-fifth longer than peristomal projection. Average length (fixed material, 11 specimens) 76μ (71–83μ), average width 27μ (20–31μ). Macronucleus club-shaped situated as in *C. panesthiae*. Micronucleus relatively large (3·5–5μ), ovoid, lying dorsal to the macronucleus. Karyophore present, consisting of a single continuous membrane attached to the right margin towards the posterior end.

Description: This species is conspicuous for its pronounced peristomal projection, the end of which opens out into a funnel. Numerous food inclusions are found in the posterior endoplasm as well as clusters of minute micro-organisms. The karyophore, unlike that of previously described species, is made up of a single membrane. The anterior and posterior endoplasms are not separated near the left margin, and often food bodies are found to have migrated in front of the nuclei. The micronucleus stains more intensely than the macronucleus and stands out sharply.

Occurrence: Eleven specimens of this species have been found so far in 1 of the 4 *Panesthia spadica* examined. It has never been found in *P. javanica*.

Type host: Panesthia spadica from Gifu, Japan.

Clevelandia parapanesthiae sp.nov. (Figs. 40–41).

Diagnosis: Body considerably flattened dorsoventrally, lateral margins evenly arched for three-fourths of the body length. Anterior end pointed. Left margin notched and recurved anteriorly toward the peristomal projection.

Average length 86 μ (65–98 μ), average width 44 μ (36–52 μ). Cytopharynx extends into body proper for a distance two-thirds to three-fourths greater than the length of the peristomal projection. Macronucleus thin, anteriorly rounded, posteriorly pointed and curved towards the right body margin. Micronucleus large (3–7 μ) usually situated ventral to the macronucleus near the mid-line. Karyophore present, consisting of a single membrane. Peristomal projection pronounced and relatively thin.

Description: This species resembles *Clevelandia panesthiae* in general body outline, except for the presence of a definite notch where the left margin meets the peristomal projection. The macronucleus is much thinner and more elongate, and the micronucleus is larger, stains more intensely and usually lies in a different position. The cytoplasm is characteristically clear with only a minimum of inclusions. The organization of the cytopharynx, although the latter is relatively longer, is the same as in *C. panesthiae*. The karyophore is similar to that of *C. nipponensis* in that it is made up of a single membrane attached near the posterior end of the right margin.

Occurrence: Clevelandia parapanesthiae occurs in the majority of specimens of *Panesthia javanica*, although rarely in great numbers. I have not found it in *P. spadica*.

Type host: Panesthia javanica from Mt. Maquiling, Luzon, Philippine Islands.

Clevelandia hastula sp.nov. (Figs. 35–36).

Diagnosis: Body spear-shaped, nearly cylindrical in cross section. Peristomal projection long, as much as one-half the entire body length. Average body length 78 μ (65–84 μ), average width 21 μ (18–28 μ). Macronucleus ovoid, situated in the anterior third of the body with its long axis slightly oblique to the long axis. Micronucleus large (3–4·5 μ), anterior to the macronucleus. Karyophore inconspicuous or absent.

Description: Clevelandia hastula is similar to *C. nipponensis* in general shape although the spear-head appearance is more pronounced owing to the greater narrowing of the body to form the peristomal projection. This is very long and relatively narrow, and is able to bend considerably as the organism swims. The macronucleus is different in shape from that of *C. nipponensis*, and the micronucleus, in addition to lying anterior to it, stains much more faintly. The macronucleus, extending nearly to the edge of the body, makes it extremely difficult to determine whether or not a karyophore is present. I have never seen any indication of one, even in sectioned organisms, and I am inclined to think that it is absent. This is indicated also by the fact that the macronuclear ends are round, not pointed as in *C. nipponensis* where they are held in by the karyophore. The lack of a karyophore might seem to indicate that *C. hastula* is in reality a newly excysted form of *C. nipponensis*; but they do not occur in the same host, and among hundreds of individuals that I have examined from *Panesthia javanica* I have found only those of the type described above.

The radiating fibres line the cytopharynx for a little over one-half its circumference.

Occurrence: Common in the hindgut of *Panesthia javanica* but apparently absent from *P. spadica*.

Type host: Panesthia javanica from Mt. Maquiling, Luzon, Philippine Islands.

Paraclevelandia gen.nov.

Diagnosis: Clevelandiidae with ovoid rigid body. Posterior end truncated obliquely to the left. No peristomal projection.

Type species: Paraclevelandia brevis sp.nov. from the hindgut of the wood-feeding roach *Panesthia*.

The genus *Paraclevelandia* is differentiated from *Clevelandia* mainly by the absence of a peristomal projection, the peristome being sunk into the truncated posterior end. In addition, the karyophore attachment is entirely different. Its single membrane is attached at both ends near the anterior end of the organism, so that the long axis of the macronucleus is nearly parallel to the long axis of the body. In the two species studied so far an additional difference may be noted. In the anterior end of the body occurs a sac-like organ surrounded by the karyophore. It is always present in *simplex* and usually present in *P. brevis*. I have evidence (to be published later in connexion with endomixis) that this sac is intimately connected with the macronucleus and serves as a reservoir for old macronuclear chromatin and possibly for its release. In material stained with the Feulgen reagent the contents of the sac are coloured a faint lavender.

Paraclevelandia brevis sp.nov. (Figs. 42–43).

Diagnosis: Small ciliate, somewhat flattened dorsoventrally, pointed anteriorly and obliquely truncate posteriorly. Average length 28 μ (16–38 μ), average width 19 μ (9–21 μ). Macronucleus ovoid to sausage-shaped, relatively large and situated at the anterior end, with its long axis slightly oblique to the long axis of the body. Micronucleus very large and situated in the mid-region of the body dorsal to the macronucleus. Karyophore present, consisting of a single membrane attached to the anterior end of the body.

Description: This is the smallest species of ciliates found in *Panesthia*. The peristome is wide and occupies the greater width of the truncated posterior end. The dorsal lip of the peristome is somewhat longer than the ventral, thus forming a flange. The membranelles are few and very conspicuous. The radiating fibres are but slightly arched, and line only the dorsal third of the cytopharynx. The macronucleus is variable in shape, being usually ovoid but sometimes elongate or nearly spherical. Its usual position is indicated in the figures (Figs. 42–43), but when spherical it often lies in the exact body centre. The micronucleus stains very intensely with iron-haematoxylin and remains black after the macronucleus has been completely decolorized. The anterior sac-like structure is usually conspicuous but occasionally inconspicuous or

apparently absent. When present its contents stain a faint grey with haematoxylin and a faint lavender with the Feulgen reagent.

Occurrence: This species was present in 100 per cent of the *Panesthia javanica* examined and the incidence of infection is probably nearly as high in *P. spadica*. In numbers it exceeds any of the other species of ciliates present. Owing to its small size, however, it is not as strikingly noticeable as some of the other forms.

Type host: Panesthia javanica from Mt. Maquiling, Luzon, Philippine Islands.

Additional host: Panesthia spadica from Gifu, Japan.

Paraclevelandia simplex sp.nov. (Figs. 44–45).

Diagnosis: Body pointed anteriorly and tending to be evenly rounded posteriorly, flattened dorsoventrally. Average length 79μ (72–88μ), average width 39μ (30–46μ). Body surface deeply grooved. Ectoplasm very thick, containing irregularly shaped refractile bodies lined up between the grooves. Swimming motions characteristically slow and steady. Peristome wide. Macronucleus elongate, sausage-shaped or bar-shaped, lying with its long axis parallel to the long axis of the body, towards the right margin. Micronucleus small, lying at about the level of the anterior third of the macronucleus. Karyophore present, similar to that in *Paraclevelandia brevis*.

Description: This is one of the most striking ciliates present in *Panesthia* because of the striations on the body surface and the very conspicuous ectoplasmic bodies arranged in the pattern of the surface grooves. Its shape is constant and the wide peristome and cytopharynx contain large and conspicuous membranelles. The radiating fibres extend in the same manner as those of *Paraclevelandia brevis*. The macronucleus is characteristically thin and rod-like, lying near the right body margin. The micronucleus is smaller than that of *P. brevis* and stains more faintly. It often lies to the right of the macronucleus, usually dorsal to it. Its position seems to be more variable, however, than in other ciliate species found in *Panesthia*. The anterior sac-like structure is always present and very conspicuous. *P. simplex* regularly undergoes endomixis just before encystation.

Occurrence: This species has never been found in great abundance in either of its hosts, although the incidence of infection is about 50 per cent in *Panesthia javanica*.

Type host: Panesthia javanica from Mt. Maquiling, Luzon, Philippine Islands.

Additional host: Panesthia spadica from Gifu, Japan.

Cysts

(Fig. VIII)

Various types of cysts are continually encountered in the intestinal contents of *Panesthia javanica*. I have examined them in life, in material stained with Feulgen's reagent, and in sectioned material stained with Heidenhain's

haematoxylin and Mallory's triple stain, yet I am still unable to say, with a few exceptions, which cysts belong to which species of Clevelandiidae. When these ciliates loose their typical organization and encyst it is difficult to

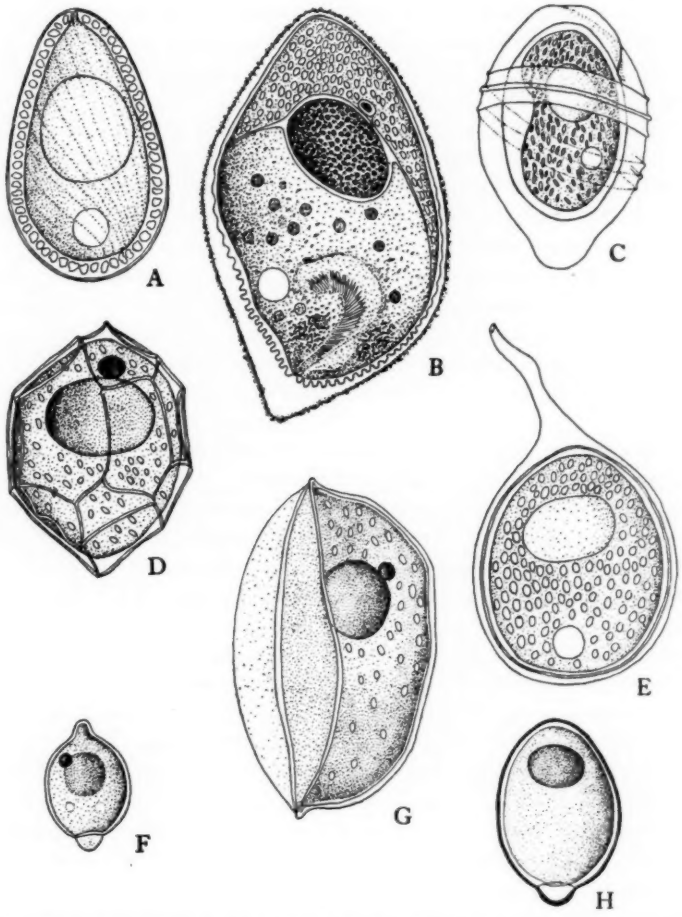

Fig. VIII. Various types of cysts found in *Panesthia javanica*. D, F, and H × 1002, the remainder × 556. A, *Paraclevelandia simplex*. Life. B, *Clevelandia panesthiae*(?). C, Unidentified. D, *Clevelandia hastula*. E, Unidentified. F, *Paraclevelandia brevis*. G, Unidentified. H, *Nyctotherus uichancoi*, very small types frequently encountered.

identify them. I give illustrations of the various cysts encountered, however, and note the evidence for their allocation, though until more work is done, especially on the cultivation of these forms, no final naming of some of these cysts is possible.

Paraclevelandia simplex encysts in a highly characteristic manner. I have seen this process in life, and because this species retains its peculiar ectoplasmic refringent bodies and other features throughout, I am certain of the type illustrated in Fig. VIII A. The body striations remain distinct within the thin cyst wall, and a vacuole is always present in what was the posterior end of the trophozoite. The macronucleus is represented by a large spherical body with the chromatin in the form of a twisted thread (as in *Nyctotherus*). In stained material the micronucleus is plainly visible.

Fig. VIII B represents an organism undergoing encystation. From its size (100μ in length) and structural make-up I believe it to be a specimen of *Clevelandia panesthiae* or possibly *C. elongata*. Many particles of debris adhere to the cyst wall as though it were sticky. The peripheral cilia have all been resorbed but some of the membranelles are still present.

Fig. VIII C is a type of frequent occurrence in my material. No internal organization except the spherical macronucleus, a vacuole, and the glycogen-like platelets can be seen. The cyst wall is characteristically formed with an anterior flange and a spirally formed three-part ridge that encircles the cyst slightly more than one turn. I cannot say which species this cyst represents.

Because of its frequent occurrence in roaches heavily infected with *C. hastula*, its size (34–40μ), and its micronucleus, I believe the cyst shown in Fig. VIII D belongs to this species. The macronucleus is ovoid and the micronucleus is spherical. Glycogen-like platelets are scattered throughout the endoplasm. The cyst wall is crenulated in a distinctive pattern. I have encountered cysts of this type frequently in both living and stained material.

Fig. VIII E represents a peculiar type, often seen, that I cannot identify. Apart from the internal structures (macronucleus, vacuole and glycogen-like platelets) there appear to be two distinct cyst membranes. One is smooth and surrounds the protoplasm while the other is drawn out into a long tube, which appears to be open to the exterior. Both cyst walls are rather thick and yellowish in life.

What is undoubtedly the cyst of *Paraclevelandia brevis* is represented in Fig. VIII F. This is the most frequently encountered cyst in *Panesthia*, is the smallest (14–19μ), and contains no glycogen bodies, as one would expect in this species. There is an ovoid macronucleus and a conspicuous micronucleus. The cyst wall is smooth and colourless. It is drawn out at the two ends into knobs, one of which possesses a cleft forming an operculum as in *Nyctotherus uichancoi*.

The dish-shaped cyst represented in Fig. VIII G has been encountered frequently but I cannot say to which species of ciliate it belongs. The protoplasm, containing the nuclei and the glycogen-like platelets, is concentrated in the heavy walled portion of the cyst (the dish). Covering the dish-shaped portion is a delicate membrane which is very transparent.

GENERAL CONSIDERATIONS

It is becoming more clear as the work on the Protozoa associated with different insects progresses, that any consideration of phylogenetic relationships of the hosts should take into consideration their guests, at least in species of hosts that lead a relatively secluded existence. Termites and roaches of the wood-feeding species are all more or less isolated from other closely related species owing to their habits. On the other hand, the common cockroaches, *Periplaneta* and *Blatta*, are cosmopolitan in their distribution and mingle freely. It is not surprising, therefore, that no significant differences have been observed in the intestinal fauna of these two genera. As several workers have pointed out (Kirby, 1927; Cleveland *et al.* 1934) the termites are closely related to the blattids as evidenced by some of the genera of intestinal Protozoa found in both. Kirby draws attention to the similarities between *Endamoeba blattae*, from *Periplaneta* and *Blatta*, and the large xylophagous amoebae of *Mirotermes*, especially *Endamoeba simulans*. He also notes the affinities between the small cockroach-amoeba *Entamoeba thomsoni* (Lucas, 1927, mentioned independently in the same year in a footnote on p. 209 of Kirby's work) and *Endamoeba disparta*, also from *Mirotermes*. He points to this as evidence that the infections of the domestic cockroach and *Mirotermes* most probably were derived from ancestors common to both insects. Cleveland *et al.* (1934) draws attention to the fact that the hypermastigote flagellates are entirely confined to roaches and termites. Until the publication of his monograph on *Cryptocercus* the sole representative of this group in cockroaches was thought to be *Lophomonas*. But the similarities of the hypermastigotes of *Cryptocercus* and termites are striking, and, as Cleveland points out, this adds weight to the belief that the two groups (blattids and termites) have had a common origin.

Further data were obtained in the genus *Amitermes* by Kirby (1932). He found, in addition to small polymastigote flagellates and an *Endamoeba*, a species of the genus *Nyctotherus* that resembled in many respects *N. ovalis* from the domestic cockroach. But representatives of this genus are found in such a wide variety of hosts that its occurrence would seem to be of no phylogenetic significance. However, when the isolated habits of the termites are taken into consideration it must be admitted that the likelihood of contamination infection is reduced, and that this case falls into the same category with the hypermastigote flagellates and the amoebae mentioned above.

The relationships between *Cryptocercus*, *Panesthia*, and the domestic cockroaches are seen in their general morphology. In food habits *Cryptocercus* and *Panesthia* are similar, and distinct from the omnivorous domestic cockroaches. But when the protozoan faunas are taken into consideration *Panesthia* resembles more closely the domestic cockroaches than it does *Cryptocercus*. In both are found species of *Endamoeba*, *Monocercomonoides*, *Hexamita*, and *Nyctotherus*, while in *Cryptocercus* only *Monocercomonoides* and *Hexamita*

occur. The genus *Lophomonas*, common in the domestic cockroach, is not found in *Panesthia*. In addition to the fauna of the common domestic cockroach, there are found in *Panesthia* members of the new family Clevelandiidae, which do not occur in *Cryptocercus*. These forms are unquestionably closely related to the genus *Nyctotherus* and they have probably been evolved from common ancestors. But the separation of the Clevelandiidae from *Nyctotherus* must have taken place at a later date than the separation of the hosts, otherwise we should expect to find representatives of the family in *Periplaneta*, *Blatta*, etc. Or it may be that the wood-feeding habits of the host influenced the evolution of the Clevelandiidae. This does not appear to be the correct explanation, however, since members of this family are entirely absent from *Cryptocercus* (a wood-feeder).

Wenrich (1935) in a recent paper dealing with host-parasite relationships argues that the evolution of protozoan species within a given host most probably has taken place through mutations and that natural selection has determined the survival value of the structural or physiological modifications produced. He believes that mutations may arise spontaneously or may be stimulated by some definite environmental agency. If the mutation was one that benefited the organisms it persisted, such as the hooks on the gregarine *Actinocephalus* and the ciliate *Hoplitophyra*. One would have to assume, if Wenrich's hypothesis is to be generally accepted, that the stimulating forces of the environment need not act equally on all individuals, and the mutations, when they arose, would need only to be not detrimental for survival.

In *Panesthia* the evolution of the Clevelandiidae appears to have progressed along definite lines in the same environment and under the same conditions in which the genera *Monocercomonoides*, *Hexamita*, *Endamoeba* and *Nyctotherus* have remained relatively static. And the type of evolution is not one that can easily be explained as purely adaptive. It is difficult to see what advantage, if any, it is to these ciliates to possess a posterior mouth. On the other hand, considering their rather slow swimming movements and the abundance of food (bacteria) at all times it does not appear that the posterior mouth is less efficient than the lateral one of *Nyctotherus*. It might be claimed that here was an illustration of directive or orthogenetic evolution, persisting in spite of the lack of direct benefit. Kofoid (1935), in a description of two Ophrioscolecids from the Indian elephant, discusses the probability of the orthogenetic evolution of these forms as regards the multiplication of membranelles, the excretory apparatus, the spiral condition and the body metamerism. He believes that the evolution of the two genera described (*Polydinium* and *Elephantophilus*) has taken place along with the evolution of their host. This last supposition, if applied to the roaches, would seem to place the genus *Panesthia* (because of the Clevelandiidae) higher in the evolutionary scale than *Periplaneta* or *Blatta*, contrary to the accepted belief, and would fail to take into consideration that only minute structural changes have occurred in the other protozoan genera.

It seems probable, I believe, that members of the Clevelandiidae have evolved from a *Nyctotherus*-type, perhaps in response to a peculiar environmental situation, not equally active, however, on the other protozoan genera living as their associates.

As regards the association of the protozoa making up the intestinal fauna of *Panesthia* with the host, it is probable that most if not all of the species should be considered as harmless commensals. There is nothing to indicate that any of the species harm the host, and with the exception of *Endamoeba javanica* no evidence that any benefit is derived by the host. Because of its xylophagous habits *E. javanica* may be instrumental in preparing some of the ingested cellulose for use by the cockroach. Kirby (1927) suggested this possibility in the case of the amoebae of *Mirotermes*. But the small numbers of amoebae occurring in any one *Panesthia*, as compared with *Mirotermes*, would certainly preclude the possibility of any but infinitesimal benefit to the host. That *Panesthia* depends upon an entirely different type of organism to prepare its cellulose for assimilation was demonstrated by Cleveland *et al.* (1934) when he isolated cellulose-digesting bacteria from its foregut. Cultures of these organisms were found capable of digesting cellulose rapidly *in vitro*.

SUMMARY

1. The Protozoa from the hindgut of the wood-feeding roaches *Panesthia javanica* and *P. spadica* are described.

2. These Protozoa are to be regarded as commensals.

3. They belong to three subphyla of the phylum Protozoa.

4. Two are flagellates, *Monocercomonoides panesthiae* sp.nov. and *Hexamita cryptocerci* Cleveland.

5. Two species of *Endamoeba* are described, *E. philippinensis* sp.nov. and *E. javanica* sp.nov., the former small and a bacteria feeder, the latter large and xylophagous.

6. An unidentified amoeba was observed in one roach.

7. *Nyctotherus uichancoi* sp.nov. was found in the majority of *Panesthia javanica* and infrequently in *P. spadica*.

8. Nine species of heterotrichous ciliates (*Clevelandia* gen.nov. and *Paraclevelandia* gen.nov.) make up the family Clevelandiidae fam.nov.

9. This family is characterized by the posterior position of the mouth which may occur at the end of a projection, varying in length. It is closely related to the family Plagiotomidae, to which *Nyctotherus* belongs.

10. A well-integrated neuromotor system is described in the family Clevelandiidae, being practically identical in all members.

11. The relationships of *Panesthia*, *Cryptocercus*, the domestic cockroaches, and the termites are discussed on the basis of their protozoan faunas.

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EXPLANATION OF PLATES VIII TO X

All figures are of whole organisms except Figs. 26, 31, 32 and 38. With the exception of Fig. 4 they were all made with the aid of a camera lucida, from material stained in Heidenhain's haematoxylin. Various destaining agents were employed and these will be indicated by the following abbreviations: A., 2 per cent iron alum; P., saturated aqueous picric acid; H., hydrogen peroxide; HCl, acidulated 70 per cent alcohol. Abbreviations for the fixatives used: S., Schaudinn's fluid; B., Bouin's fluid; F., Flemming's fluid.

PLATE VIII

Figs. 1–8. *Monocercomonoides panesthiae* sp.nov. ×3000.

Fig. 1. Elongate form showing the clear axostyle, two posterior stained bodies and a deeply staining sphere just posterior to the nucleus. B., A.

Fig. 2. Elongate form possessing only one large posterior body, stained sphere and rhizoplast connecting the nuclear membrane and the blepharoplast. B., A. Note the crescent-shaped cytostome.

Fig. 3. Spherical form. B., A.

Fig. 4. Elongate form drawn from life. Note the axostyle extending from the posterior end of the body.

Fig. 5. Elongate form showing the protruding axostyle with adhering particles of debris. S., A.

Fig. 6. Early division stage with two daughter blepharoplasts and an intradesmose. S., A.

Fig. 7. Anaphase of mitosis. S., A.

Fig. 8. Telophase showing the reorganizing daughter nuclei and the two new axostyles.

Figs. 9–11. *Hexamita cryptocerci* Cleveland *et al.* ×3000. S., A.

Figs. 12–15. *Endamoeba philippinensis* sp.nov. ×2000.

Fig. 12. Large form. Note the many bacteria-like organisms contained in the endoplasmic vacuoles. B., P.

Fig. 13. Form similar to Fig. 12 except smaller. B., A.

Fig. 14. Uninucleate cyst. Note heavy cyst wall. S., A.

Fig. 15. Binucleate cyst with two chromatoid bodies. S., A.

Figs. 16–19. *Endamoeba javanica* sp.nov. Trophozoites. ×2000.

Fig. 16. Individual destained to show the granule within the chromatin mass. Many refractile wood particles in the endoplasm. Note definite ectoplasmic layer. B., P.

Fig. 17. Large individual showing the characteristic folding of the nuclear membrane. Also a number of peripheral nucleoli. B., P.

Fig. 18. Nucleus in early stage of mitosis. S., A.

Fig. 19. Probably a metacystic amoeba. Two small (*nn*) nuclei and one large (*N*) nucleus. S., A.

Fig. 20. Cyst of *Endamoeba javanica* sp.nov. with two nuclei. The nucleoli are large. Note the absence of wood particles. S., A. ×1500.

PLATE IX

Fig. 21. *Nyctotherus uichancoi* sp.nov. Dorsal view. B., P. ×403.

Fig. 22. *Nyctotherus uichancoi* sp.nov. Ventral view. B., P. ×403.

Fig. 23. *Nyctotherus uichancoi* sp.nov. Ventral view of small organism, probably just excysted. Note spherical macronucleus and absence of karyophore. F., H. ×816.

Fig. 24. *Clevelandia panesthiae* gen.nov., sp.nov. Ventral view. B., A. ×403.

Fig. 25. *Clevelandia panesthiae* gen.nov., sp.nov. Dorsal view. S., A. ×403.

Fig. 26. Longitudinal section through the peristomal projection of *Clevelandia panesthiae* gen.nov., sp.nov. showing the dorsal tips of the basal plates of the membranelles and the radiating fibres in the roof of the peristome and cytopharynx. This is a ventral view. 5μ section. F., A. ×583.

Fig. 27. *Clevelandia contorta* gen.nov., sp.nov. Dorsal view. B., A. ×403.

Fig. 28. *Clevelandia contorta* gen.nov., sp.nov. Left lateral view. B., A. ×403.

Fig. 29. *Clevelandia elongata* gen.nov., sp.nov. Ventral view. B., P. ×403.

Fig. 30. *Clevelandia elongata* gen.nov., sp.nov. Dorsal view. S., A. ×403.

Fig. 31. Cross section 5μ thick, through the cytopharynx at the region of the motorium, of *Clevelandia elongata* gen.nov., sp.nov. Note the basal plate, the ectoplasmic thickening, the cross section of the submembranellar fibres, the radiating fibre and the motorium. In this section the top is the ventral surface of the organism and the section is viewed from the anterior end. B., A. ×583.

Fig. 32. From the same organism as Fig. 31. Section taken through the peristome.

PLATE X

Fig. 33. *Clevelandia nipponensis* gen.nov., sp.nov. Ventral view. S., A. ×816.

Fig. 34. *Clevelandia nipponensis* gen.nov., sp.nov. Dorsal view. Note the large and deeply staining micronucleus and the karyophore attached to one side of the body only. S., A. ×816.

Fig. 35. *Clevelandia hastula* gen.nov., sp.nov. Dorsal view. Note ovoid macronucleus, faintly staining micronucleus and absence of karyophore. B., P. ×816.

Fig. 36. *Clevelandia hastula* gen.nov., sp.nov. Ventral view. B., HCl. ×816.

Fig. 37. *Clevelandia constricta* gen.nov., sp.nov. Ventral view. B., P. ×816.

Fig. 38. Longitudinal section, 5 μ thick, through the peristome of *Clevelandia constricta* showing similar structures to those in Fig. 26. B., A. ×1116.

Fig. 39. *Clevelandia constricta* gen.nov., sp.nov. Dorsal view. In this organism the continuation tube is visible, also what is probably a frontal lamina. B., P. ×816.

Fig. 40. *Clevelandia parapanesthiae* gen.nov., sp.nov. Ventral view. Note deeply staining micronucleus. S., P. ×403.

Fig. 41. *Clevelandia parapanesthiae* gen.nov., sp.nov. Dorsal view. B., A. ×403.

Fig. 42. *Paraclevelandia brevis* gen.nov., sp.nov. Dorsal view. Note the anterior sac-like structure and the karyophore attached near the anterior end. B., P. ×1024.

Fig. 43. *Paraclevelandia brevis* gen.nov., sp.nov. Ventral view. B., P. ×1024.

Fig. 44. *Paraclevelandia simplex* gen.nov., sp.nov. Ventral view. The characteristic thick ectoplasm containing the refractile bodies are shown here in optical section. S., A. ×816.

Fig. 45. *Paraclevelandia simplex* gen.nov., sp.nov. Right lateral view. S., HCl. ×816.

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TWO NEW SEMI-PARASITIC COPEPODS FROM THE PERUVIAN COAST

By CHARLES BRANCH WILSON
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(With 17 Figures in the Text)

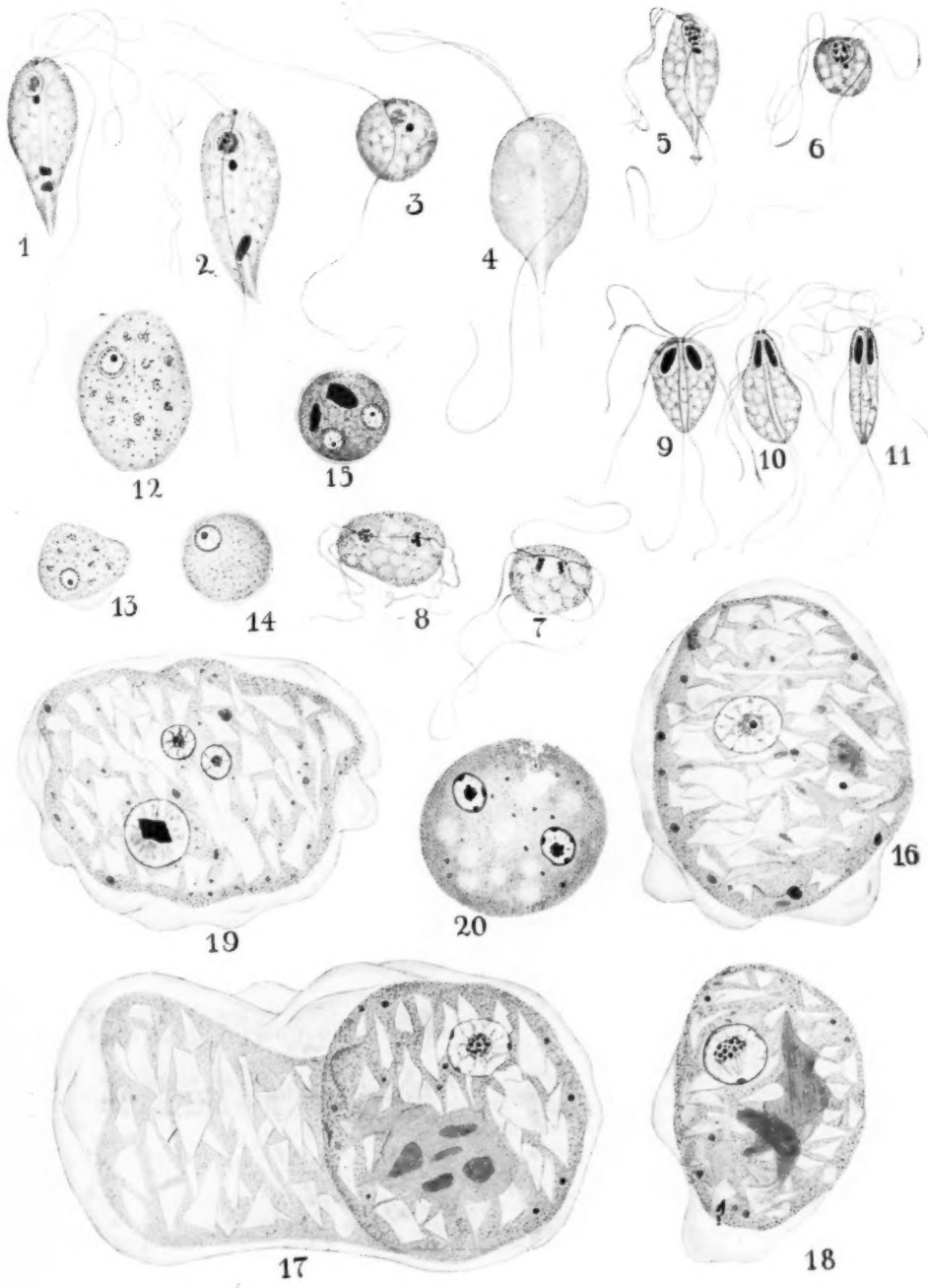
Among the shore crabs the genus *Callianassa* is quite cosmopolitan; its species are widely distributed and seem also to be peculiarly susceptible to parasitism. Wherever they have been examined carefully they have yielded semi-parasitic copepods, sometimes in considerable numbers. Such forms have been reported from both the east and west coast of North America, from the Mediterranean and from the British Isles. Recently 150 of these semi-parasites were sent to the United States National Museum from Salaverry near Trujillo on the coast of northern Peru. They were taken by Mr Floyd Sears, agent of the Grace Line at Salaverry, from the branchial chamber of a species of *Callianassa* on the Peruvian coast. They prove to be new species belonging to two genera of cyclopid copepods and are here described and figured.

Hemicyclops elongatus n.sp.

(Figs. 1–6)

Occurrence. Species belonging to this genus are often captured while swimming freely in the plankton, and nothing is known of their host. A species on the Californian coast was found crawling over the egg masses of ovigerous *Callianassa* females. In this instance a single female was taken among the specimens of *Clausidium* swarming in the branchial chamber. Of necessity it becomes the type of the new species and has been given Cat. No. 60,431, U.S.N.M. The male is still unknown.

The female. Body much elongated and very slender; cephalothorax one-fourth longer than wide, narrowed anteriorly and with the posterior corners bluntly rounded and scarcely projecting. Second, third and fourth segments narrowed regularly, with their epimeral plates expanded laterally and closely imbricated. Fifth segment abruptly reduced to half the width of the fourth, with projecting lateral margins. Urosome nearly as long as the metasome, very slender and made up of five segments. Genital segment shorter than the basal abdominal segment, considerably dilated through the centre and narrowed anteriorly and posteriorly; oviduct openings lateral, each with two setae. Basal segment of abdomen longest, twice the length of the third segment, which is the shortest. Caudal rami narrow cylindrical, four times as long as wide, narrowed at the tip, with four apical setae, the two middle



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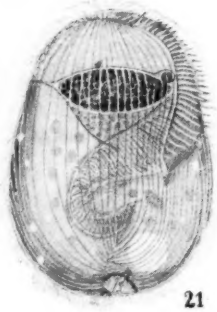

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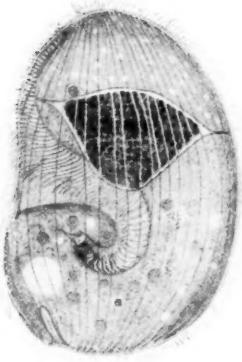

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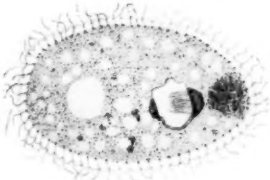

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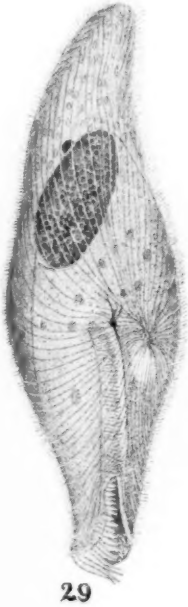

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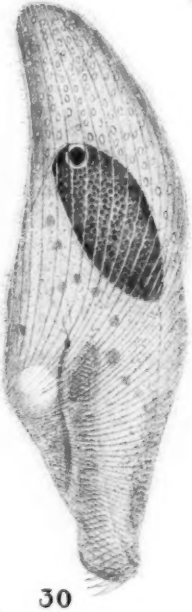

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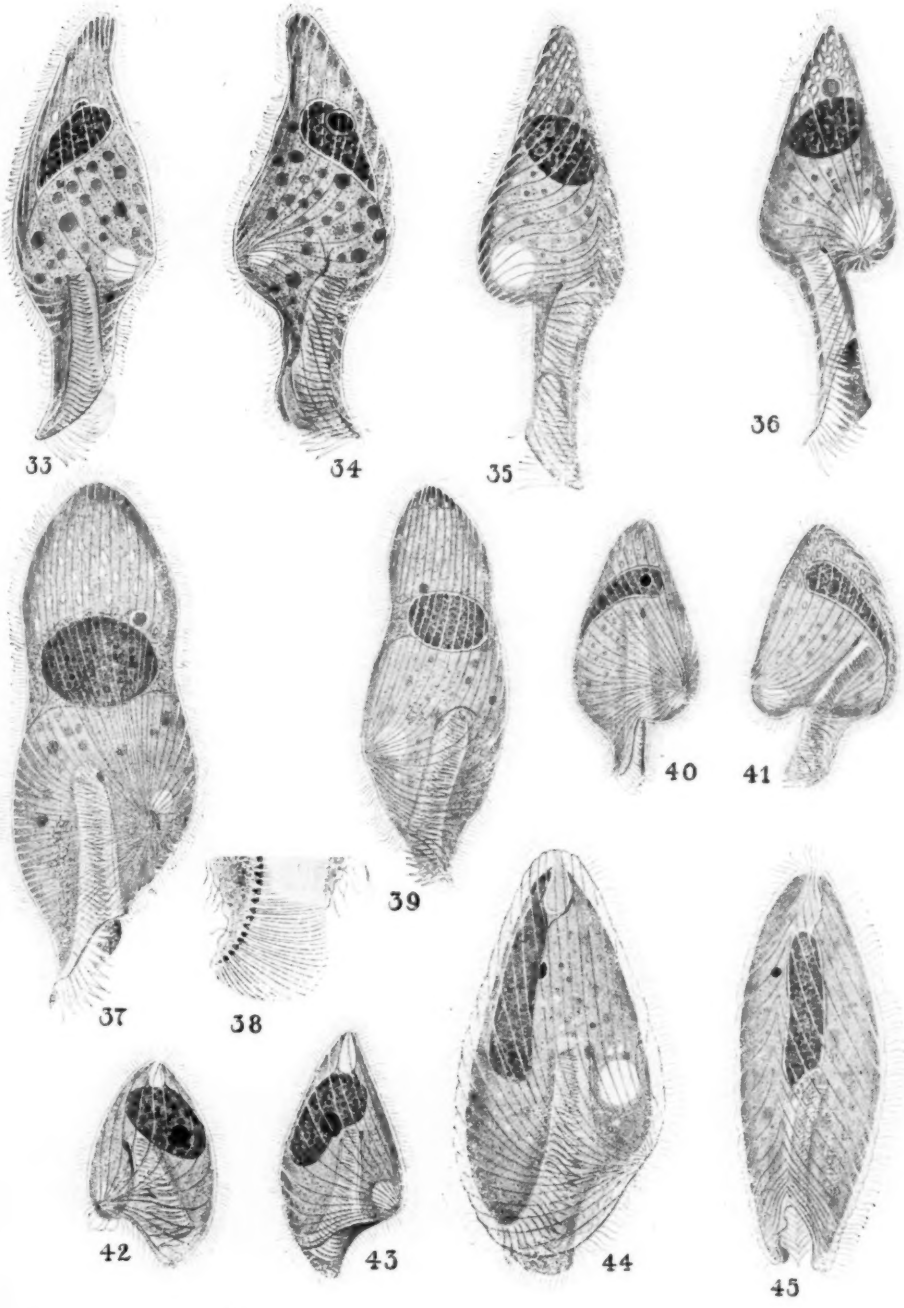

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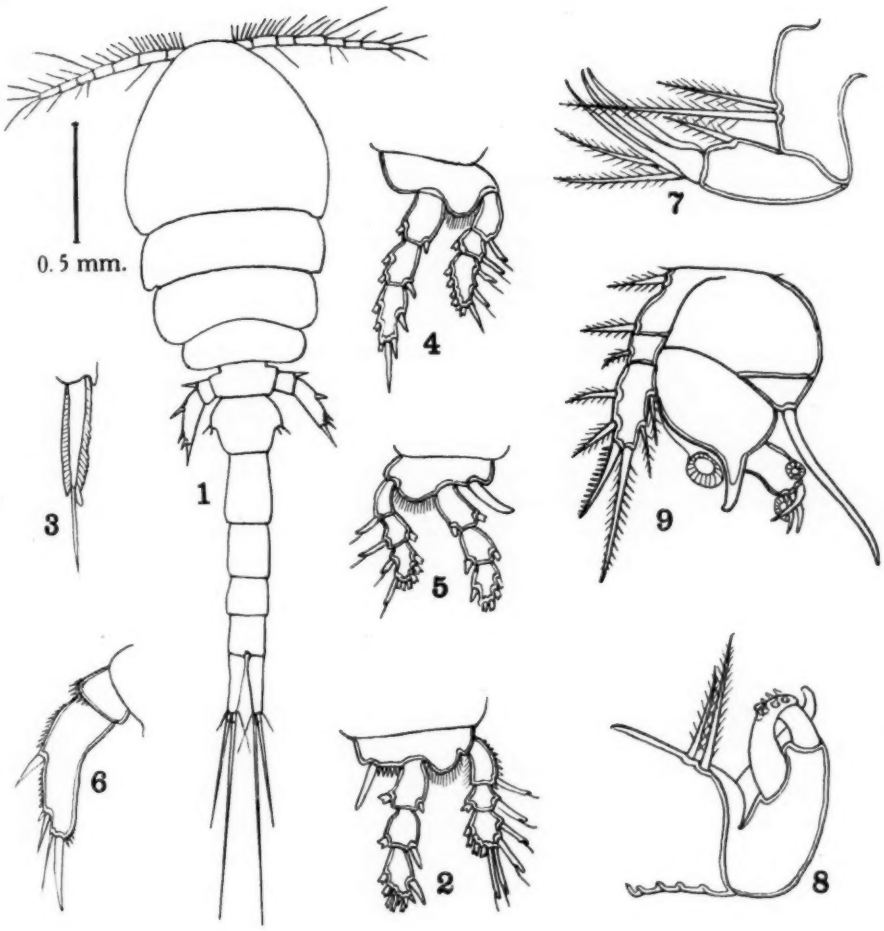

Fig. 1. Dorsal view of *Hemicyclops elongatus*.
Fig. 2. First leg.
Fig. 3. Spine of first expod, enlarged.
Fig. 4. Second leg.
Fig. 5. Fourth leg.
Fig. 6. Fifth leg.
Fig. 7. Maxilliped of female *Clausidium scarsi*.
Fig. 8. Maxilliped of male.
Fig. 9. First leg of male.

ones enlarged and jointed near the base, the inner of the two as long as the whole abdomen.

The first antennae are seven-segmented, the segments of varying lengths; the second antennae are four-segmented, and, with the mouth-parts, are similar to those in other species of the genus. In the first legs the two basal endopod segments have each a single inner seta and an outer spine at the distal corner. The end segment has two setae on the inner margin, three much shorter ones at the tip, a spine on the outer margin and a row of four short spines around the tip above the bases of the setae. The outer and apical spines of the exopod have membranous margins, the one on the distal side notched near the tip and carrying there a bristle (Fig. 3). The basal segment is serrate on its outer margin, the end segment has two outer and two apical spines, and four inner setae.

In the fifth legs the basal segment is much wider than long and is armed at its outer distal corner with a row of short spines instead of a seta. The distal segment is bent near the middle, the outer margin and the distal end of the inner margin are serrate, and there are four spines, one terminal and three on the outer margin, the proximal one near the centre. Total length 3 mm. Width of cephalothorax 0·90 mm.

Remarks. The species can be identified by its body proportions and the details of the legs: the mouth parts are left intact in the single specimen.

Clausidium searsi n.sp.

(Figs. 7–17)

Occurrence. Every species of this genus thus far obtained has been found within the branchial chamber of some species of *Callianassa*, and often in considerable abundance. Although it seems probable that they may come out at times and swim about in the plankton this has never been verified. More than 150 specimens were obtained, the numbers of the sexes being about equal. Every female carried a male fastened to her abdomen as in Fig. 10 and they remain in that condition in the preservative. A single pair, the female with egg strings, have been selected to serve as types of the new species, with Cat. No. 60,434, U.S.N.M.

The female. Head fused with the first segment, the cephalothorax one-third wider than long with pointed epimeral plates at its posterior corners; antennal area well defined but no eye visible. Second and third segments as wide as the cephalothorax, but together little more than half as long, each with pointed epimeral plates at the posterior corners. Fourth and fifth segments fused and covered with a single dorsal plate, almost circular in outline. This plate is two-thirds as wide and long as the cephalothorax; its anterior half is fused with the dorsal surface of the thorax, while its posterior half is free and projects backward over the genital segment nearly to the centre of the latter. The centre of the plate is raised a little dorsally into a small circular

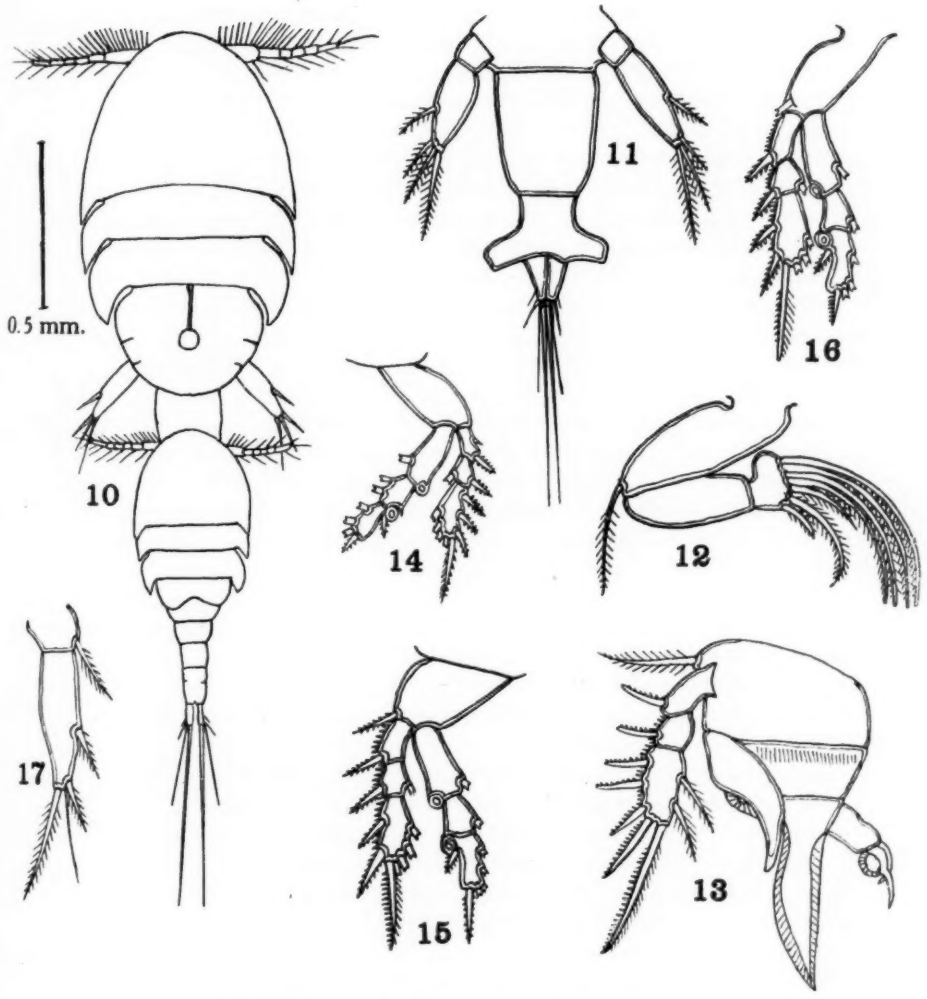

Fig. 10. Dorsal view of female and attached male of *Clausidium searsi*.
Fig. 11. Fifth and genital segments and abdomen of female.
Fig. 12. Second antenna.
Fig. 13. First leg of female.
Fig. 14. Second leg of female.
Fig. 15. Second leg of male.
Fig. 16. Fourth leg of male.
Fig. 17. Fifth leg of female.

coloured area, from which a sort of keel or rib runs forward to the anterior margin of the plate. There are also two slight incisions on each lateral margin, the anterior ones opposite the central raised area.

The genital segment is about a fifth longer than wide, tapered somewhat posteriorly, with nearly straight sides. The abdomen is made up of a single segment, whose posterior corners project laterally in the form of a sub-rectangular process on either side, extending outward at right angles to the body axis. These processes are grasped by the maxillipeds of the male and furnish an attachment firm enough to persist even in preservatives. The caudal rami are juxtaposed and twice as long as wide, each tipped with three setae with a shorter one on the outer margin near the tip.

The first antennae are seven-segmented, the second segment the longest and the fourth the shortest, the three basal segments densely setose. In both living and preserved specimens and in both sexes these first antennae almost always extend outward in line with each other and at right angles to the body axis and hence are visible in dorsal view. The second antennae are three-segmented, the basal segment as long as the other two combined with a long seta at its outer distal corner. The terminal segment is narrowed at its base and much widened at its tip which is armed with a row of seven curved setae, the four inner ones equal and longer than the three outer ones which are unequal.

The first maxilla is made up of a short process armed with small setae, the second maxilla is biramose, the endopod divided at its tip. The maxilliped is composed of two stout segments, the basal one with a pair of long unequal setae on its inner margin near the centre. The distal segment has a single seta on its inner margin near the distal end and an apical tuft of five setae, three of which are curved and claw-like.

In the endopod of the first legs the spine at the inner distal corner of the basal segment is two-segmented, the end segment wide, lanceolate in shape, with a membranous margin on each side and extending beyond the tip of the longest seta of the exopod. Across the base of this spine is a transverse row of long hairs. The spine of the second segment is an elongate cone, slightly curved and projecting outside of the spine on the basal segment. No spine is visible at the base of the process on the third segment but there is a large sucking disk beneath, and mostly concealed by, the spine on the second segment. This third segment process is turned inward and tipped with two unequal claws beneath which is another large sucking disk with a ribbed membranous border. The exopod of the first leg is three-segmented, the two basal segments each with one serrate spine, the end segment with three outer serrate spines, two longer unequal terminal spines pectinate on one margin, and two inner setae. In the second, third and fourth legs the middle endopod segment has two inner setae and the distal segment has three setae in the second and third legs, but only two in the fourth leg. Each endopod of these three pairs of legs is also armed with two sucking disks, one on the first and

one on the second segment at the outer distal corner. Each fifth leg is made up of a short basal joint more or less fused with the thorax and carrying a seta at its outer distal corner, and a distal segment three times as long as wide. This segment is narrowed toward the tip and is armed with one outer and three apical setae, the middle one of which is filiform. Total length 1·60 mm. Width of cephalothorax 0·66 mm.

The male. Body considerably smaller and more slender than that of the female; cephalothorax as long as wide with sharp epimeral plates at the posterior corners. Second and third segments narrowed a little but with similar epimeral plates. The dorsal plate covering the fused fourth and fifth segments is re-entrant posteriorly and just overlaps the anterior margin of the genital segment. The latter is divided at the centre and has no rudiments of a sixth pair of legs, unless a spine at each posterior corner might represent them. The caudal rami are twice as long as wide, but neither tapered nor juxtaposed as in the female.

The antennae and mouth parts are like those of the female except the maxillipeds, which are greatly modified for prehension. The basal segment is wider than long and is armed on its inner margin with two long and stout setae. The distal segment is tipped with three stout curved claws, two of which form a powerful chela, their outer margins armed with stout spines, while the third shuts diagonally against them. When the male grasps the processes on the abdomen of the female the chela gives it so firm a grip that the two can only rarely be separated without injury.

In the first legs the spine at the distal corner of the basal segment is very slender and cylindrical, but as long as in the female. The spine on the second endopod segment is much wider and longer than in the female, and the process on the third segment is also longer, with similar sucking disks. The other swimming legs are like those of the female, except that the middle segment of all the endopods has but a single seta. Total length 0·90 mm. Width of cephalothorax 0·33 mm.

Remarks. This increases the number of species in the genus to six, but like the other five this species also was found in the branchial chamber of the decapod crab, *Callianassa*. It may be readily distinguished from any of the other species by the protuberances on the abdomen of the female and by the area in the centre of the dorsal shield on the fused fourth and fifth segments.

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THE OCCURRENCE OF *ONCHOCERCA GUTTUROSA* NEUMANN IN CATTLE IN ENGLAND, WITH AN ACCOUNT OF ITS LIFE HISTORY AND DEVELOPMENT IN *SIMULIUM ORNATUM* MG.

By J. S. STEWARD

(With Plate XI, containing Figs. 1–10)

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I. Introduction

Nematode worms of the genus *Onchocerca* Diesing 1841 have been found in connective tissue of the cervical ligament and spleen of cattle in Cambridgeshire and Herefordshire. The specific diagnosis seems to lie between *O. gutturosa* Neumann 1910 and *O. bovis* Piettre 1912. As the resemblance to the former species is the closer it is proposed to regard the parasites examined as *O. gutturosa* with the probability that *O. bovis* is a synonym. Furthermore, it seems not unlikely that the reference of Stiles (1892) to "long thin worms in the connective tissue between the spleen and stomach of cows", named by him *Filaria lienalis* refers to this species; but as the description remains so vague the matter cannot be decided in the absence of the type material of *F. lienalis*.

Slight but constant differences exist between the cuticle and size of specimens from the spleen and those from the cervical ligament, as may be seen in Table I.

Microfilariae typical of *Onchocerca* were found in the skin of infected cows, and in investigating the life cycle of the parasite it was found that complete development of the microfilariae takes place in *Simulium ornatum* Mg.

II. The mature worms

The adult worms of both sexes are generally found more or less closely intercoiled together in a small area of yellowish golden gelatinous exudate lying between the two lamellar portions of the ligamentum nuchae where it is widest, i.e. opposite the second to fourth cervical vertebrae, and otherwise

more or less as described by M. Emery and published in Neumann's account (1910) of *Onchocerca gutturosa*.

The English material examined scarcely gave the impression of "veritables petites tumeurs molles" as described by Emery, though there was a diffuse soft swelling due to infiltration of the connective tissue with the straw-coloured exudate; possibly the description of M. Emery applies to heavier infestations of the ligament than were found in the English cattle.

Table I.

Males

	O. bovis Piettre	*O. gutturosa* Neumann	Specimens from cervical ligament	Specimens from spleen
Length (cm.)	4–5·5	—	2·9–3·9	3·2
Maximum thickness (mm.)	0·085–0·095	0·091–0·105 (av. 0·098)	0·095–0·115	0·07–0·085
Oesophagus (mm.)	0·75–0·80	0·95	1·01–1·12	0·89–1·0
Anterior end-inflation (mm.)	—	0·4	0·25–0·3	—
Width at inflation (mm.)	—	0·08	0·079–0·1	—
Width before inflation (mm.)	—	—	0·055–0·70	—
Small spicule (mm.)	0·180–0·210	0·225–0·295 (av. 0·250)	0·182–0·25	0·2–0·23
Large spicule (mm.)	0·065–0·075	0·075–0·088 (av. 0·080)	0·078–0·09	0·065–0·08

Females

	O. bovis Piettre	*O. gutturosa* Neumann	Specimens from cervical ligament	Specimens from spleen
Length (cm.)	Above 26 (not determined)	Above 55	Up to 60 or more	Up to at least 35
Maximum thickness (mm.)	0·26–0·29	Av. 0·3	0·2–0·315	0·15–0·26
Length of oesophagus and width (mm.)	0·82–0·85	1·15	1·09–1·35 ×	1·25–1·38 × 0·065–0·075
Anterior extremity—inflation (mm.)	—	0·09–0·1	0·25–0·37	—
Width in front of inflation (mm.)	—	(and behind 0·081)	0·062–0·085	—
Width at centre of inflation (mm.)	—	—	0·079–0·11	—
Anterior extremity to vulva (mm.)	0·63–0·65	0·55	0·72–0·89	0·55–0·63
Tail length (mm.)	—	—	0·19–0·24	—
Cuticular thickenings—depth (mm.)	—	Cuticle is 0·035–0·047	0·015 (max.)	0·004 (max.)
Cuticular thickenings distance apart (mm.)	3 internal striations	0·09–0·110	0·2	0·065
Microfilariae (mm.)	0·23–0·265 × 0·0055	0·17–0·195 × 0·004	0·2–0·312	0·25–0·312
Embryonated eggs (mm.)	0·048–0·053 × 0·036	0·035–0·045 × 0·028–0·035	0·031–0·052 × 0·031–0·046	0·044–0·058 × 0·033–0·052

The situation of the worms in the connective tissue on or between the flat surfaces of the ligamentum nuchae (lamellar portion), as compared with the position of *Onchocerca cervicalis* in the actual substance of the ligament in horses, may account for the relative absence of tissue change due to the parasites in cows. Figs. 1 and 2 illustrate this difference.

Infestation of the spleen occurs in the connective tissue joining that organ to the rumen, and it is best detected in fresh specimens by excising the spleen

with a portion of rumen attached, and carefully separating the spleen from the rumen. The female worms can then be seen as white thread-like parasites winding about in the connective tissue. Some specimens appeared to be fixed by growth of fibrous connective tissue into one position so firmly that they were almost impossible to extricate. Others shewed evidence of calcification. It is thought that those surrounded by connective tissue are younger than the fibrosed or calcified specimens, being possibly the infection of one or more summers later.

Infestation of the spleen was usually less than that of the ligamentum nuchae. Males were very difficult to see on account of their extreme thinness, being best seen as a minute ridge when looked at from an obtuse angle against the light. The best method of extracting the worms was to insert a fine needle under the middle of their body and draw them out; sometimes it was first necessary to pierce the connective tissue above them. A few males were extracted intact, but complete females were never obtained though fragments of the latter over 25 cm. long were sometimes extracted. Other methods attempted were maceration in water at 37° C., or digestion with either pepsin solution or calf-stomach extract, but these were not found satisfactory. Maceration failed probably because the soft connective tissue surrounding the parasites appeared to be the last tissue to be digested; whereas with *O. cervicalis* in the substance of the ligamentum nuchae of horses, maceration was more successful because the fibres fell apart leaving the worms easier to extract than in the case of *O. gutturosa*.

Female worms from the cervical ligament show a continuous spiral ridge-like thickening of the cuticle, which commences at a short distance from either extremity and increases in size, prominence and spacing towards the middle of the body. This cuticular thickening is undulating, as may be seen in Fig. 3, although Neumann (1910) and Piettre (1912) both state that the thickening is not undulated.

In specimens from the gastro-splenic connective tissue the cuticular thickenings were much less marked than in worms from the neck; the thickenings did not exceed 4·5 μ in depth.

In the English specimens from both the neck and the spleen considerable variations occurred in the papillae of both sides; it would seem, therefore, that the papillae are of little value for specific diagnosis.

A further slight difference between the specimens from the neck and spleen was the increase in diameter near the anterior extremity; this was well marked in the cervical specimens while it was less easily detectable in specimens from the spleen. Photomicrographs at the same magnification illustrate this (Figs. 5 and 6).

It is possible that the cervical ligament is a more natural and suitable habitat than the gastro-splenic connective tissue, and the difference in the consistency and amount of the connective tissue of the two sites may be sufficient to account for the more or less constant variations in the worms obtained from the two different situations.

III. THE MICROFILARIA

Microfilariae occur in the skin of infected cows at a depth of about 1 mm. from the surface.

The microfilariae are almost identical with those of *O. cervicalis* from horses; the only observed difference was that the widths of the bovine microfilariae were slightly greater than in those from horses. Table II shows measurements of (*a*) microfilariae from the skin of cows, and from the uterus of adult females from (*b*) the cervical ligament, and (*c*) gastro-splenic connective tissue of cattle and also of (*d*) *Microfilaria cervicalis* from the skin of horses.

Table II

		Length μ	Maximum width μ	Distance from anterior extremity to nerve ring μ	Width at nerve ring μ	Distance from anterior extremity to 1st nucleus μ	Distance from posterior extremity to last nucleus μ
(*a*) Microfilariae from skin of cows	Range	150–220	4–6	40–50	3·5–5	4–5·24	8
	Av.	(192·7)	(4·9)	(44·7)	(4·1)		
(*b*) Microfilariae *ex utero* of females from ligamentum nuchae		243–260 (248)	4·16 (4·16)	55–64 (60)	—	—	—
(*c*) Microfilariae *ex utero* of females from gastro-splenic connective tissue		235–266 (256)	4–6 (4·66)	58–62 (61)	—	—	—
(*d*) *Mf. cervicalis* from skin of horse		160–217 (198)	3·5–4	35–52 (44·8)	2–4 (2·66)	3–5·5	6–9

It may be seen that the microfilariae *ex utero* are considerably larger than those from the cow's skin. However, only a few specimens of the latter were obtained, and they were not fixed so freshly as the microfilariae obtained *ex utero*. If it had been possible to examine a longer series, it is probable that they would have covered a wider range and more closely approximated the *ex utero* specimens.

IV. THE LIFE CYCLE

In investigating the life history of the parasite dissections were made of as many specimens as possible of the various species of blood-sucking flies which could be obtained after feeding on an infected cow. Most of these were negative; for instance, 86 engorged specimens of *Stomoxys calcitrans* were all negative, as also were 10 specimens of *S.* (*Lyperosia*) *irritans*. Four specimens of *Musca* sp. which had fed on blood drawn by other flies were also free of microfilariae.

With regard to *Culicoides nubeculosus*, which had been previously shewn (Steward 1933*b*) to act as the vector of *Onchocerca cervicalis* of horses, 106 specimens were caught feeding on the infected cow. These were dissected and four were found to contain developmental (sausage-shaped) forms of a micro-

filaria although they had fed less than 24 hours previously on the cow. From the advanced stage of their development it was obvious that these microfilariae were taken up in a previous meal, possibly on a horse infected with *O. cervicalis*. One midge of this species, however, contained an undeveloped (skin) form of microfilaria which, of course, might be either from the infected cow or a recent feed on a horse harbouring *Microfilaria cervicalis* (*Onchocerca cervicalis* was actually known to occur within a short distance of the farm, but the horses on the farm where the investigation took place were not examined for the presence of this worm).

In order to know definitely whether *Culicoides nubeculosus* became infected with microfilariae from infected cows, a large number of the midges were hatched in the laboratory in the following year and fed on the infected cow. None of the 101 that fed to repletion acquired microfilariae, so it was concluded that *C. nubeculosus* is not a suitable intermediate host for *Onchocerca gutturosa*. Of other species of *Culicoides* four specimens of *C. stigma* and *C. obsoletus* were found to be free from microfilariae on dissection.

Flies of the genus *Simulium* were not abundant on the farm where this investigation was pursued (Bottisham Fen in Cambridgeshire), probably because of the absence, in fen country, of the swiftly running streams in which *Simulium* generally breeds. In the course of 3 weeks less than 50 flies of this genus were caught there, whereas in more favourable (hilly) districts as many as 100 may sometimes be caught in one hour. Nevertheless, of 48 *S. ornatum* that had fed on the cow and were examined by sectioning or dissection, no less than 21 contained microfilariae (42 per cent). This species appears to act as a most efficient vector, thus accounting no doubt for the occurrence of *Onchocerca* in cattle in districts where *Simulium* is relatively scarce. Although it was impossible to obtain infected flies in large numbers, a series of specimens was secured illustrating all stages of development in the fly. (Figs. 7, 8, 9 and 10.)

Development of *Microfilaria gutturosa* in *Simulium* resembles that of *Microfilaria cervicalis* in *Culicoides* described elsewhere (Steward *loc. cit.*), and will only be described briefly here.

The microfilariae are conveyed from the skin with the blood of the cow to the mid-gut of the fly, and there development commences. At first the length decreases and width increases and within a few days migration forwards into the thoracic muscles takes place. About 10 days after ingestion by *Simulium* the width of the microfilaria, which is now in the so-called "sausage" stage in the thoracic muscles, has increased from about 5μ of the skin forms to between 18 and 22μ, while the length may still be about 200μ.

Subsequently the length of the microfilaria begins to increase to an observed maximum of 650μ at 15 days after ingestion, the width varying from 10–30μ and being greatest in the posterior third.

Between the 19th and 22nd day the microfilariae migrate forwards to the head and appear to be ready for emergence by the proboscis when the infected flies feed again.

The earliest infective microfilariae observed in the head were in flies dissected 19 days after feeding on the cow. It is believed that infective microfilariae may lie in the anterior portion of the thorax just posterior to the head and migrate into the proboscis at the time of feeding on a suitable host.

It was hoped to obtain development of the microfilariae in laboratory-reared *Simulium* in the following summer (1934) but, under the circumstances, it was impossible to obtain the required species of *Simulium*. Certain species of *Simulium* were, however, reared in the laboratory and induced to feed on the infected cow: unfortunately adequate numbers were obtained of only two species, these were *S. salopiense* and a variety of *S. reptans*, 173 and 122 engorged flies of these species were dissected, but none had become infected. It appeared from examination of portions of the cow's skin that microfilariae were much less numerous in the skin in 1934 than in 1933; this, of course, may have prejudiced the chance of infecting the flies considerably. In addition, 20 engorged specimens of *S. erythrocephalum* were dissected—one of these which had fed three days previously on the infected cow contained a living microfilaria in which the width had attained 7 μ and the length 240 μ; it appears that development of this microfilaria had begun and that this species may possibly act as a suitable host.

V. Discussion

The genus *Onchocerca* is composed of a number of species of parasites of the connective tissue of man and animals. Relatively little is known of the relationships of the different species of the genus, and the descriptions of most of the species are fragmentary. It is not unlikely, however, that when knowledge of the life histories of the various species is available, it may be possible to make a more accurate classification of the genus.

At the moment, the only life cycles known are those of *O. volvulus* of man, which Blacklock (1926) in West Africa shewed was carried by *Simulium damnosum*, and *Onchocerca cervicalis* of horses which was shewn (Steward, 1933*b*) to be carried by *Culicoides nubeculosus*. There is no information on the life cycles of any of the numerous species parasitic in cattle, although there have been numerous investigations in Australia during the past 20 years to find the carrier of *Onchocerca gibsoni*. That bovine Onchocerciasis is of considerable economic importance in Australia may be realised by the statement in a Commonwealth of Australia publication (1917) that the direct loss from "worm nodules" due to *Onchocerca gibsoni*, to the State of Queensland alone, was estimated at £500,000 per annum.

It is hoped that the demonstration of the life cycle of *O. gutturosa* in *Simulium* will facilitate the discovery of the carrier of *Onchocerca gibsoni* which is of such importance in Australia.

VI. Summary

1. *Onchocerca* is recorded for the first time from English-bred cattle.

2. A specific diagnosis of *O. gutturosa* is made, *O. bovis* being regarded as a synonym. It is suggested that Stiles's description of *Filaria lienalis* may have referred to this species.

3. The microfilariae occur in the skin of infected cows, and blood-sucking flies of the genus *Simulium* were found to be the vectors.

4. Complete development was observed in *S. ornatum* and partial development in *S. erythrocephalum*. *S. ornatum* appears to be a very efficient intermediate host, as over 40 per cent became infected.

5. *Culicoides nubeculosus*, the vector of *Onchocerca cervicalis* of horses, does not appear to carry the bovine *Onchocerca*.

Acknowledgements

The writer is deeply indebted to Dr H. A. Baylis, of the British Museum (Natural History), for assistance in the examination and classification of the adult worms, and to Dr F. W. Edwards, also of the Natural History Museum, for identification of the species of *Simulium*. This opportunity is also welcomed to record the writer's gratitude and indebtedness to Mr John Towler of Tonbridge Hall, Bottisham, Cambridgeshire, who so kindly lent an *Onchocerca*-infected cow for the duration of the experiment and provided every possible facility throughout the investigation.

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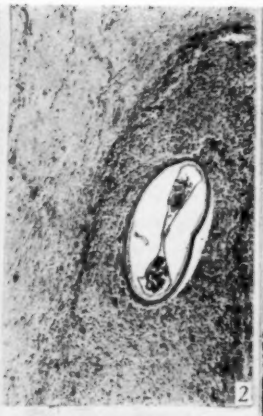
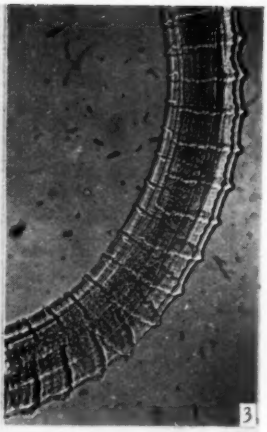
EXPLANATION OF PLATE XI

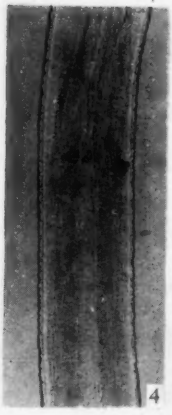
Fig. 1. Lamellar portion of ligamentum nuchae of cow shewing cross sections of *Onchocerca gutturosa* in the connective tissue on the medial surface. ×40.

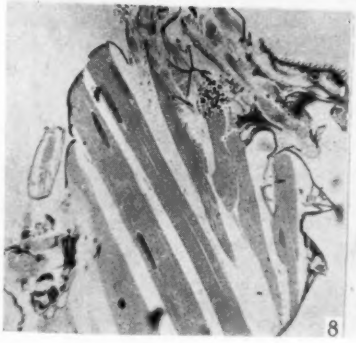
Fig. 2. Section of ligamentum nuchae of a horse (suffering from Fistulous Withers) shewing *O. cervicalis*. In contrast to *O. gutturosa* in the cow the parasite is in the substance of the ligament and surrounded by an inflammatory reaction.

Fig. 3. Female *O. gutturosa* from connective tissue on the ligamentum nuchae of a cow. ×50.









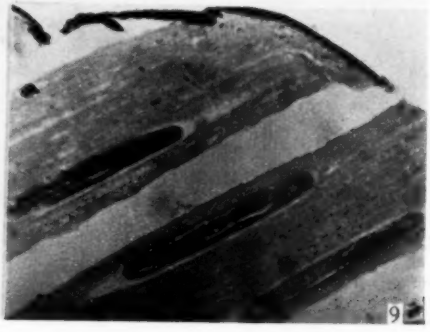


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Fig. 4. Female *O. gutturosa* from the gastro-splenic connective tissue of a cow. ×50.

Fig. 5. Anterior extremity of a male *O. gutturosa* from connective tissue on the ligamentum nuchae of a cow. ×90.

Fig. 6. Anterior extremity of a male *O. gutturosa* from the gastro-splenic connective tissue of a cow. ×90.

Fig. 7. *Microfilaria gutturosa* from the skin of a cow. (Leishman's stain). ×400.

Fig. 8. Longitudinal section of *Simulium ornatum* 13 days after feeding on an infected cow. Four developmental forms of *Microfilaria gutturosa* can be seen in the thoracic muscles.

Fig. 9. A higher magnification of portion of Fig. 8.

Fig. 10. Dissection of the head of *Simulium ornatum* 21 days after feeding on an infected cow. An infective form of *Microfilaria gutturosa* is seen unstained. ×approx. 90.

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ON THE FUNCTION OF THE POLAR FILAMENT OF *NOSEMA BOMBYCIS*

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(With 6 Figures in the Text)

Among the new facts discovered in the course of the writer's investigation on the function of the polar filament of the spore of *Nosema bombycis* (Ohshima, 1927), the following two are considered the most important:

(1) As soon as its filament extrusion is accomplished in the midgut of a silkworm, the spore discharges a viscous fluid through the tube of the filament. This fluid remains hanging in a spherical shape at the extremity of the filament.

(2) Both the polar filament and the viscous fluid are speedily digested by the digestive ferment of the silkworm in a few seconds.

As was pointed out previously (Ohshima, 1927), the latter phenomenon renders dubious the hypothesis advanced by Bütschli, Thélohan, Doflein, and others that the filament is an attachment apparatus; and further inquiries have at last brought to light the astonishing fact that the viscous fluid is the amoebula itself. The following is a brief note of the experiment:

Observations in the haemolymph of the silkworm

As it was almost impossible to observe the germs with exactitude in the digestive fluid on account of their immediate digestion, investigations were concentrated on inventing an effective method. After many years of vain attempts, a very successful method for evagining the polar filament was at last discovered, and it has since been used exclusively in the observation of the germ. When the spore is treated with alkali or acids for a reasonably long time so that it may not be killed, and when the temperature is kept approximately between 23 and 30° C., its filament is observed to evaginate instantly at the suitable range of *p*H where the sample is neutralized. For instance, if NaOH is used as reagent, and if the duration of time is limited to an hour, the available concentration range of NaOH becomes from $N/10$ to $N/160$, and the available *p*H range of neutralization becomes from 6·0 to 9·0, though the optimum points are a little narrowed in both cases. But it is unquestionably an unphysiological method of filament evagination, not adequate for the present purpose. So the writer tried to apply the haemolymph of silkworms to it. Normally, the haemolymph is inactive to spores, but it has a strong buffer action and is available as a neutralizing reagent, its *p*H value being 6·5. Thus it becomes possible to carry out the evagination in the haemolymph. The

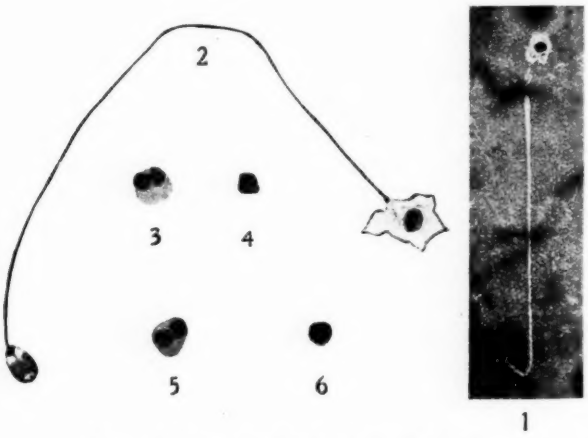
method is as follows: after previous treatment of spores with NaOH, the sample is neutralized with HCl of concentration equal to that of the base to an extent a little higher than *p*H 11, the upper limit of filament evagination. Then a small quantity of it is added to an excess of haemolymph. This second neutralization may be done either on a slide or in a test-tube. The results of such experiments are quite satisfactory as shown in the following.

Observation of fresh material. Observation was made under the dark field microscope, magnifying about 1000 times. Filaments begin to evaginate immediately after the sample is mixed with haemolymph and in a few minutes nearly all the spores have their filaments extruded. The velocity and manner of the evagination and the shape of the substance flowing out are generally similar to those in the digestive fluid (Ohshima, 1927). The size of the outflow is also nearly the same in both cases, estimated at about 2μ. The only difference in the present case is that neither the filament nor the outflow is digested by the ferment of the haemolymph. The motionless filament is attached to the spore, and has the outflow on its tip for a long time. In the inner part of this substance there appears a hyaline-like shining portion, and again in the centre of this portion frequently comes in sight a pair of relatively large granules, though the high refractive minute granules which show active molecular motion are less in number than in the digestive fluid. This pair of granules is also highly refractive and comparatively motionless. They often look like an independent mass surrounded by a thin film which contains no granule. When the outflow is fixed on a slide, they become even more distinct and can be seen in every fixed substance, although the other contents disappear, thus suggesting that in all probability they are two nuclei of a germ. But in spite of the long observation lasting for 30 min., the writer was unable to see the creeping out movement of the germ either from the outflow or from the spore to which the filament was connected.

OBSERVATION OF STAINED MATERIAL

Smears in which the spores had germinated were stained with Giemsa's fluid, after drying and fixation with Schaudinn's fluid modified by Woodcock. When the material is stained, the empty filament remains unstained, while the haemolymph which surrounds it and its outflow shrinks remarkably. It leaves a clear space behind on drying, thus facilitating the inspection of the relation between the germ and the spore. It is clearly seen at once that the germ lodges in the outflow, as is shown in Figs. 1 and 2. Accordingly, there is no doubt that the germ is shot out from the spore through the filament. Contrary to the fresh condition, however, in the stained preparation no granule can be seen, a very large clear space existing between the germ and the contracted haemolymph (Figs. 1 and 2). Probably, this space is filled with the fluid which surrounds the germ and serves to facilitate its discharge through the tube of the filament. It is observed in a well differentiated preparation with fully

spread cytoplasm that each germ just shot out has always two nuclei (Figs. 1, 2 and 3). The results obtained by Feulgen's method of nuclear staining also testify to the existence of two nuclei both in the spore and in the germ, confirming that the germ is derived from the spore. But when the germ does not broaden its cytoplasm it appears to be mononuclear, as shown in Fig. 4. In such a case, it takes a spherical form and its cytoplasm is hardly visible, indicating the scantiness of plasma compared with the nuclei. Hence in order to measure the size of the germ, spherical individuals with unspread cytoplasm should be chosen. An experiment shows that it is 1·27 μ in diameter on an average of fifty individuals.



Figs. 1–4: Spore immediately after germination.
Figs. 5 and 6: 15 hours after subcutaneous injection.
Fig. 1, ×850; Fig. 2, ×1800; Figs. 3–6, ×3500.

Next, experiments were carried out both *in vivo* and *in vitro* to ascertain whether the germ multiplies or not in the haemolymph of silkworms. The results deny its multiplying power, showing that the germ remains unchanged (Figs. 5 and 6)—*in vitro* for any length of time and *in vivo* while it exists.[1] For example, the size of germs with unspread cytoplasm, taken from silkworms kept for 15 hours after subcutaneous injection, is 1·34 μ in diameter on an average of fifty individuals, showing no change from that in the previous case. Stempell (1909) says with regard to the fate of the germ of *Nosema bombycis*, that autogamy occurs immediately after germination, producing a planont, and that the planont, passing through the wall of the alimentary canal into the haemocoele, multiplies actively there. But, as stated above, the results of the writer's experiments disagree with his, and show that autogamy must be performed in the interior of the cell, if it takes place after germination.

[1] *In vivo* it disappears from the haemolymph in about 25 hours.

INFECTION EXPERIMENTS

In spite of the fact that no change in the germs is detected in the haemolymph, subcutaneous injection experiments manifest clearly that they have a very strong infective power. Moreover, it is revealed that when the injection is done subcutaneously with excess of spores, the worms subjected to examination die soon. In this case only such organs and tissues as are adjacent to the inoculated place are found seriously affected, the other greater parts being almost free from parasites, as shown in Table I.

Table I. *Subcutaneous injection of spores*

Individual numbers	Date of death after injection	Tissues in the neighbourhood of injection (Left boundary between VIII & IX segments)				Tissues in the non-injection part (Part anterior to V segment)			
		Silk gland	Hypo-dermis	Fatty tissue	Muscle	Silk gland	Hypo-dermis	Fatty tissue	Muscle
1	5th day	⋕	⋕	∞	⋕	—	±	—	—
2	6th day	∞	⋕	∞	∞	—	—	—	—
3	,,	⋕	∞	⋕	∞	—	—	—	—
4	,,	∞	∞	#	∞	—	—	—	—
5	,,	∞∞	∞	∞	∞	—	⋕	⋕	∞
6	,,	∞∞	⋕	∞	⋕	—	#	#	—
7	,,	—	—	⋕	—	—	—	—	—
8	,,	∞∞	∞∞	∞	∞∞	—	—	—	⋕
9	7th day	∞∞	⋕	⋕	∞∞	#	±	—	—
10	8th day (Jaundice)	Whole body posterior to V segment; #				Whole body anterior to V segment; extremely rare			
11	17th day (In the cocoon)	Whole body posterior to V segment; ∞∞				Whole body anterior to V segment; ∞			

Remarks. Silkworms examined: Japanese race, No. 110, first day of fifth stage. Spores: previously treated with *N*/50 NaOH for an hour, and then partially neutralized with N/50 HCl. Date of injection: 8 June 1932.

—, no parasite; ±, less than a parasite per field; +, 2–10 parasites; #, 11–30; ⋕, 30–100; ⋕, 101–300; ∞, more than 300; ∞∞, whole field filled with parasites.

Though twenty-three silkworms were used in both of the injected and non-injected lots, the other worms of the former were used in another study which is not written here. The non-injected worms are all free from *Nosema*. Among them thirteen worms have grown to moths.

Microscopical examinations were done with fresh smears taken at random from the parts mentioned above. Only two worms, Nos. 10 and 11, were examined by grinding the dissected body and adding a little of 2 per cent. KOH solution.

In the infection experiments per os with ordinary spores on the starved and non-starved silkworms, no difference of infectivity can be recognized, though the spore-germinating power in the case of the starved is remarkably stronger. This may be somewhat strange at first sight. Yet if it is taken into considera-

tion that effective germination can occur in both cases only in the places quite near the intestinal wall where the conditions are most favourable for infection, and that consequently the germination in both cases is nearly the same in absolute quantity, the above phenomenon will come to be looked upon as not unnatural.

It was already shown by the writer (Ohshima, 1927) that the filament can readily pierce through tissues or make its way through narrow spaces.

From these facts, it may probably be concluded that the germ is directly injected by the filament into the tissue.

At any rate, with reference to the function of the polar filament, nothing can be made out to support the suggestion of attachment apparatus. In this respect, Korke's statement (1916) of conductance apparatus in the case of *Nosema pulicis* may be said to be in agreement with the writer's results, though it has not been accepted by others, as it was described too briefly.

Conclusion

Based on the experiments mentioned above, the function of the polar filament of *Nosema bombycis* may be explained in the following way.

The polar filament of *N. bombycis* is a germination tubule. In order to germinate the amoebula in the alimentary canal, the spore evaginates its filament through the peritrophic membrane into the epithelium, and the germ is safely poured out there through the tubule of the filament, protected on the way from the digestive ferment of the canal.

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ON THE LIFE HISTORY AND THE BIOLOGY OF THE RAT-FLEA, *NOSOPSYLLUS FASCIATUS* (BOSC.)

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I. Introduction

The present work was undertaken with the idea of throwing some further light on certain aspects of the life history and the biology of fleas and to determine whether the blood-sucking insects depend for the necessary supply of iron for their normal growth on the blood or not. Fleas are one of the very few insects which live on blood throughout their life cycle and thus are a very suitable subject to experiment upon in order to find out if blood-sucking insects have taken to the blood-sucking habit because they lack iron, which is essential to their normal growth, in their body. It is experimentally proved, as will be clear from the following account, that flea larvae cannot live without blood as a part of their food and if the blood is deprived of substances which contain iron they cannot grow.

II. Material and technique

In the breeding of fleas methods described by Leeson (1932, p. 25) were employed. The humidity of the air inside the desiccators was controlled by a mixture of sulphuric acid and water in proportions given by Buxton & Mellanby (1934, p. 171). For conducting the experiments the same methods were followed as were employed by Sikes (1931, p. 245). The larvae were reared at a constant temperature of 23° C. and 80 per cent relative humidity was found most suitable for breeding experiments. A higher relative humidity of 90 per cent was also tried and no appreciable difference was observed between the results obtained with this and those with 80 per cent relative humidity; but, on the other hand, it was found that the desiccators having the atmospheric humidity of 90 per cent were often attacked by mites belonging to the species of *Tyroglyphus siro* (Linnaeus) which rarely infested the desiccators which had the atmospheric humidity of 80 per cent.

The different kinds of food were mixed with a comparatively large quantity of sand which was previously thoroughly washed with sulphuric acid and then autoclaved. All possible precautions were taken to avoid any other organic substance coming in contact either with the sand or with the food. Previous workers always ignored these precautions and hence the mixing media they employed cannot be considered free from organic substances and their experiments in this respect cannot be considered reliable.

For my experiments I have always used completely unfed and recently hatched first stage larvae, a precaution not observed by most previous workers. The pregnant females were put with crumpled pieces of black papers in a jar. After 48 hours the adults were removed and the black papers with eggs and dried defaecated blood adhering to them were transferred into another larger clean jar in which the pieces of black papers with eggs were allowed to remain loosely packed so that the larvae would drop to the bottom of the jar without having the opportunity to eat the dried blood lying close to them. In this way I was successful in getting a very large number of white larvae which were unfed and recently hatched. The fed larvae, which are always red to black-red in colour, were not used.

The blood and its different constituents were dried by evaporation in a vacuum desiccator over concentrated sulphuric acid under reduced pressure. The autoclaved substances used in these experiments were exposed to 120° C. temperature under a pressure of 15 lb. for about half an hour. In all these experiments except when otherwise stated horse blood and its various constituents have been used.

III. Historical

The earliest work on the biology and the life history of a flea is that of Leewenhoeck (1683, p. 78). He succeeded in obtaining adults from older fully fed larvae, but the newly hatched larvae, for lack of proper food, died on the twelfth day. According to Cestone (1699, p. 42), flea larvae "feed themselves on the Bran-like Substance which sticks in the Combs when Puppies are combed to take out the Fleas, or with certain Downy Substance that is found in the Plyes of Linnen Drawers or other such like Excrement". Thus he incidentally discovered for the first time the actual food of a flea larva. Rösel (1749) successfully bred recently hatched flea larvae on the blood taken from the body of mosquitoes or pigeons. Defrance (1824) suggested that the dark grains found close to the eggs were not the excreta of fleas but were particles of dried blood coming from the wound of the host after the bite of the adult flea and that they formed the main food of the flea larva. Westwood (1848, p. 318) thought that the larvae, on account of their remarkably powerful jaws, feed upon hairs, particles of wool or feathers found in places frequented by the host of the adult flea.

Laboulbène (1872, p. 270) succeeded in rearing adults from the larvae of *Ctenocephalides felis* (Bouché) fed on the sweeping of the floor which according to him did not contain blood. His supposition "que ces larves vivent de la

toison plutôt que du sang qu'elles puiseraient, où plutôt dont elles provoqueraient la sortie avec leurs mandibules" has no foundation. Blanchard (1868, p. 631) believed in the maternal instincts of fleas in regurgitating recently sucked blood into the mouth of the larvae. This fact was experimentally disproved by Pérez (1894, p. 239) by rearing larvae of the cat-flea away from their mother on dust from the bed of the host, which contained dried blood.

According to Künckel (1873, p. 140) the larvae of *Nosopsyllus fasciatus* feed on the black grains of dried blood which are in reality the excreta of the adult fleas and other organic substances present in the bed of the host of the adult fleas. Thus he was the first to describe the real nutritional requirements of the flea larvae. Mitzmain (1910*a*, *b*) gives an account of the life history and bionomics of *Pulex irritans* Linnaeus, *Xenopsylla cheopis* (Rothschild), *Ceratophyllus acutus* Baker, *Nosopsyllus fasciatus*, *Ctenocephalides canis* (Curtis) and *Leptopsylla segnis* (Schöncherr) [=*L. musculi* (Dugès)]. Patton & Cragg (1913, p. 460) found that the flea larvae had blood in their intestine "in stages varying from fresh unclotted to particles of blood pigment". According to them the adult fleas defaecate while feeding or shortly after and they pass out fresh red blood through the anus as they take up more food than they can digest. This blood is discharged in considerable quantities among the debris under the host and is ingested by the larva. Bacot & Ridewood (1914, p. 161) also considered that the chief food supply of the larvae of most species is the excreta of their parents. Bacot (1914) showed experimentally that larvae of many species of fleas require blood in their food and will not grow without it. In nature it is supplied by the adult fleas, which swallow more blood than they can digest, so that some of it is discharged along with faeces as scarcely digested blood. In 1914, Strickland described his observations on the biology of *Nosopsyllus fasciatus*.

Sikes (1930) gave a brief account of the life history of *Orchopeas wickhami* (Baker) and a detailed account of the historical survey of previous work done on the life history and the biology of fleas. In 1931 she showed that different kinds of food of flea larvae have different hygroscopic properties and the optimum atmospheric humidity differed according to the kind of food employed so as to give the optimum water content of the food used. Newly hatched larvae of *O. wickhami* were fed on different substances at varying humidity and a constant temperature, and she found that a relative humidity of 80 per cent gave the most satisfactory results.

According to Hicks (1930, p. 576), the larvae of *Tunga penetrans* (Linnaeus) developed more rapidly and with less mortality on a mixed diet of blood and other organic material. There are only two larval instars in this species.

Buxton (1931) gives a brief summary of the work done on the biology of fleas. Nordberg (1931) found that the larvae of *Ceratophyllus gallinae* (Schrank) and *C. hirundinis* (Curtis) feed for preference on the excrement of the adults, but they can survive on other organic material. Tiflov & Ioff (1932) give a detailed account of the biology and duration of the stages of the various rodent

fleas found in the south-east of European Russia at different temperatures and a constant relative humidity. It is suggested that the egg stage may represent a resting phase of the flea.

Mellanby (1933) showed experimentally that the larvae and prepupae of *Xenopsylla cheopis* are easily killed by drying but the pupae are resistant to dry environmental conditions.

IV. Life history

During the course of my work on breeding fleas for experimental purposes on the nutritional requirements of flea larvae, I found that eggs always hatched at 23° C. and 80 per cent relative humidity in 4 days' time after the adult fleas were put in jars for egg laying. There was such a constancy of the incubation period for over 10,000 eggs which I employed for my experiments that incubation period of eggs at 23° C. and 80 per cent relative humidity can safely be taken as $3\frac{1}{2}$–$4\frac{1}{2}$ days. The adult fleas were put in a jar containing black crumpled pieces of papers for 2 days and then they were removed. The hatching started after the fourth day and was completely finished after the fifth day.

There are three larval instars in this species as in most other fleas. The first stage can easily be distinguished from the other stages by the presence of a hatching spine on the head. The second and third larval stages are difficult to distinguish for lack of well-marked differentiating characters, but the size can roughly be useful in separating the two. The second stage larvae are thinner and for this reason they appear comparatively long, while the third stage larvae are thick and robust in general appearance. The larval period varies considerably, depending upon the temperature, humidity and food, and will be considered in connection with experimental work on the nutritional requirements of the flea larva. The third stage larva, before spinning a cocoon, expels the contents of its alimentary canal through the anus and one can clearly see the black contents of the alimentary canal being driven from the anterior to the posterior end. After the complete discharge of the food contents the larva becomes waxy white. This process of getting rid of the alimentary contents seldom lasts more than 4–8 hours, and this period is designated the defaecation period and it forms a distinguishing landmark between the second larval ecdysis and the last larval moult which discloses a soft white exarate pupa in which many of the external features of the imago are visible. Strictly speaking, the profound histolytic changes start immediately after defaecation, and for this reason it is advisable to divide the period from the second larval ecdysis to the final larval moult into a pre-defaecation period and a post-defaecation period.

In the beginning the defaecated larva is active and restless. After some time it starts spinning a cocoon and becomes bent in the middle so that the head and the tail ends come to lie close to each other. It soon becomes inactive and it is said to be in the resting stage. If the cocoon is opened at this stage the larva will not move and will pupate without the cocoon under suitable conditions of temperature and humidity. This resting stage has been called the

prepupa by Mellanby (1933, p. 197), a term hardly suitable for this restricted application. As a matter of fact, prepupa is an ill-defined transitional stage between the larval and pupal stages and strictly speaking it should only be applied to the pupal organization which is still within the larval cuticle and thus in outward appearance it resembles more the larva than the pupa, but it possesses all the necessary parts of the pupa although they are not fully developed. Strictly speaking, this transitional stage starts when the larva defaecates and finishes when the pupa emerges from the larval skin. Without any further moult it becomes the pupa.

If the defaecated larvae are kept in a clean tube without sand or any other kind of mixing medium and are disturbed occasionally, they will not spin cocoons and will pupate without cocoon formation. On the other hand, if not disturbed they usually migrate to the edges of the floor of the tube and each will spin an irregular web-like silky covering through which the larva is visible. I cannot agree with Mellanby's statement (1933, p. 197) that "the stimulus to spin is probably mechanical", as cocoon formation is a normal feature of flea larvae and failure of cocoon formation is mostly due to disturbances at the time of cocoon spinning, due either to some mechanical cause or to sudden changes in the environmental conditions. The absence or presence of sand or some other foreign material does not affect cocoon formation, as much as has been thought by some workers, as sometimes naked pupae are found when the larvae have been kept in sand, and cocoons, though poorly developed, are spun by larvae when kept in a clean tube.

The duration of the prepupal and the pupal periods, as pointed out by Bacot (1914, p. 474) and Mellanby (1933, p. 197), is not affected in any way whether the larva has spun a cocoon or not. Advantage was taken of this fact and larvae were made to pupate without cocoon formation in order to watch the changes that would normally take place inside the cocoon. Table I is based on observations specially made for determining the duration of the various well-defined periods during the metamorphosis of the flea larvae which were fed on their natural food. It was found that these observations agree more or less with those for larvae that were fed upon special food having all the necessary nutritional requirements of the flea larva.

Table I. *Showing the duration of various periods of metamorphosis at* 23° C. *and* 90 *per cent relative humidity*

No.	Periods of metamorphosis	Time taken
1	Recently defaecated larvae to become resting larvae	39–60 hours
2	Recently defaecated larvae to form cocoons	2–3 days
3	Resting larvae to pupate	38–73 hours
4	Recently defaecated larvae to pupate	3½–5 days
5	Pupation period	7–8½ days
6	Cocoon formation to adult emergence	10–12 days

It is clear from Table I that the time of resting and of cocoon formation is the same. The larvae as a rule are sluggish at the time of cocoon formation.

Usually when I opened a recently formed cocoon the larva was in the resting stage. According to Mellanby (1933, p. 197) a sudden increase in temperature makes the larva leave the cocoon. The sand particles are only adherent to the silky cocoon and are not interwoven in the meshes. Bacot (1914, p. 473) believed in the possibility of a larva collecting fragments of foreign material and attaching them together with silky threads. As the larva is extremely sluggish at the time of cocoon formation, it is more probable that the fragments adhere to the freshly formed sticky silk threads. The pigmentation of the adult cuticle starts within the pupal cuticle at 23° C. temperature and 90 per cent relative humidity after the fifth day of the pupal life.

According to Bacot (1914, p. 474) the larvae of *Nosopsyllus fasciatus* form hard and soft cocoons. The hard ones according to him are connected with aestivation and hibernation. During the course of my work I never came across such a thing and the cocoons were always of one kind.

V. Nutritional requirements of the flea larva

It is an admitted fact now that the larvae of various species of fleas, although they eat organic refuse indiscriminately, cannot be reared successfully unless a small quantity of dried blood is added to it. In nature the blood is supplied by the parent fleas, who swallow large amounts of blood and pass out a good deal of it in an undigested condition. Bacot (1914, pp. 472, 514, 516) showed that the dried blood was much more necessary to larvae of *Nosopsyllus fasciatus* than to those of *Pulex irritans* and *Xenopsylla cheopis*. He was successful in rearing the larvae of the last two species on crushed rat faeces alone. Sikes (1931, p. 246) successfully reared the larvae of *Orchopeas wickhami* on blood, alone or when mixed with sand, and on blood serum mixed with sand. The percentage of reared adults was low and varied considerably in her experiments. The following experiments were made in order to find out the effect of blood and its various constituents on the development of the larvae of *Nosopsyllus fasciatus*, and it was found that blood, although the most important part of the nutritional requirements of flea larvae, is not alone sufficient for their normal and successful development.

Table II. *Showing the effect of blood, its different constituents or yeast when separately mixed with acid-washed sand on the growth of flea larvae at temperature* 23° C. *and relative humidity* 80 *per cent*

Food	Newly hatched larvae used	Time within which all larvae died in days	Percentage of reared adults
Blood	91	33–40	0
Red corpuscles	151	12–21	0
Serum	70	19	0
Fibrin	106	8–20	0
Baker's yeast	100	12–15	0

The larvae when fed upon blood (Table II) were active and strong for 30 days and then suddenly they became sluggish and finally died without

forming cocoons within 33–40 days in the third stage. When fed upon red blood corpuscles they died in 12–21 days. A few died in the first larval stage, most reached the second larval stage and a few even managed to reach the third larval stage. The larvae showed signs of sluggishness from the beginning. On serum they lived for 19 days and reached the second larval stage. In the beginning the larvae fed upon serum looked healthier and stronger than those fed upon red corpuscles but in the end they were equally sluggish and died in the second larval stage. The larvae when fed on fibrin did not moult and died in 8–20 days. They all died in the first stage without any appreciable increase in their size. From the foregoing observations it is clear that the fibrin has no nutritional value; serum and red corpuscles have practically the same food value with this difference: that larvae were more active when fed on serum than when fed on red corpuscles. The blood has evidently more nutritive value than its three constituent parts when given separately.

My observations on the effect of blood mixed with acid-washed sand and blood serum mixed with acid-washed sand on the rate of development of the larvae of *Nosopsyllus fasciatus* are different from those of the previous workers. Every previous worker used mixing media which were never deprived of organic material. So I take it for granted that partial successful breeding in those cases was not entirely due to blood alone. Sikes (1931, p. 247) obtained 0–56 per cent adults by rearing larvae of *Orchopeas wickhami* on blood alone as the result of three different experiments. Such a great variation of percentage leads me to think that there was some mistake in her experiments, and that the blood she used must have got mixed with some other organic substances. This is further borne out by the fact that she got comparatively higher percentage of successfully reared adults with blood or serum on cloth than blood or serum mixed with sand; as it is probable that a piece of cloth would contain more organic substances than sand. So it appears that for the successful rearing of larvae of *O. wickhami*, the addition of some organic substances to blood is as necessary as it is in the case of the larvae of *Nosopsyllus fasciatus*. At any rate, the larvae of *N. fasciatus* cannot be successfully reared on blood alone and I am sure that if the same precautions are observed it would hold good for other species, as the mixing media used by Bacot and other workers cannot be considered free from other organic substances, which formed the accessory food.

The addition of dry baker's yeast to blood (Table III) gave almost a hundred per cent success in the rearing of fleas. The larval and pupal life was completed in 26–38 days. It is, therefore, clear that flea larvae require an accessory food in addition to blood. In nature this accessory food is supplied by organic matter present in the host's bed or in dust present in the breeding place.

Red corpuscles and yeast, when given to the larvae as a food, gave 10–50 per cent success in rearing flea larvae. The larval and the pupal life was completed in 29–45 days. Serum and yeast gave equally good results. The percentage of successful breeding was 20–55, and the duration of the larval and pupal

Table III. *Showing the effect of blood or its different constituents when separately mixed with yeast and acid-washed sand on the growth of flea larvae at temperature* 23° C. *and relative humidity* 80 *per cent*

Food	Newly hatched larvae used	Larvae which reached adult stage	Time taken to become adults in days	Percentage of reared adults	Remarks
Blood and yeast in equal parts	30	30	26	100	Minimum and maximum larval life 12 and 16 days
	35	33	33	94	
	35	35	33	100	
	20	15	38	75	
Red corpuscles and yeast in equal parts	30	3	45	10	Minimum and maximum larval life 12 and 23 days
	30	15	39	50	
	25	4	45	16	
	40	8	37	20	
	25	3	29	12	
Serum and yeast in equal parts	25	5	48	20	Two larvae in each experiment lived more than 48 days; minimum larval life 12 days
	35	9	48	26	
	20	6	34	30	
	20	11	33	55	
Pure rabbit serum and yeast in equal parts	20	4	54	20	3–6 larvae in each experiment lived more than 43 days; minimum larval life 17 days
	20	5	55	25	
	20	6	55	30	
	20	10	61	50	
	20	9	54	45	
Haemoglobin and yeast in equal parts	20	5	55	25	About 5 larvae in each experiment lived more than 40 days; minimum larval life 24 days
	20	3	43	15	
	20	5	53	25	
	20	3	54	15	
Fibrin and yeast in equal parts	95	0	—	0	All larvae died in I stage in 13–17 days

life was 33–48 days. It appears that as far as the percentage of successful breeding is concerned, the serum is equal in nutritive value to the red corpuscles, but some of the larvae prolonged their larval life to such an extent that their larval life was longer than the combined larval and pupal periods of others. Such larvae have been called by Bacot (1914, p. 524) lagging larvae. The lagging larvae seldom formed cocoons and usually died before developing into adults. I attribute this phenomenon of prolonged growth of flea larvae to the lack of some essential substance in the serum. The larvae which do not get the necessary nutritional requirements for their development, but just enough for them to live on, become lagging larvae. Pure rabbit serum and yeast when given as food to the larvae gave 20–50 per cent success in rearing flea larvae. The larval and pupal periods were prolonged and there was a comparatively higher percentage of lagging larvae. The adults obtained from larvae fed on serum were always pale yellow in colour whilst the others, obtained from larvae fed on other kinds of food, were deep brown in colour. It appears that, as regards the rate of growth, serum as a food is as good as red corpuscles; but, as regards the normal development, it has lower nutritional value than red corpuscles.

When the larvae were fed upon haemoglobin and yeast 15–25 per cent success in rearing flea larvae was obtained. The adults obtained were of extremely small size and the periods of larval and pupal life were prolonged with a fairly high percentage of lagging larvae. It seems that the nutritional value of haemoglobin is much less than that of the red corpuscles or the serum.

In fibrin and yeast larvae cannot grow and they die within 13–17 days in the first stage without any appreciable increase in size. It is therefore clear that the nutritional value of fibrin as a food for the flea larvae is practically nil.

The larvae did not grow in yeast alone (Table II) and they died within 12–15 days in the first stage without any appreciable increase in size. It seems, therefore, that yeast by itself does not fulfil the necessary nutritional requirements of flea larvae, and some blood proteins are absolutely necessary for the successful development of the flea larvae.

Table IV. *Showing the effect of different combinations of the constituents of blood when mixed with yeast and acid-washed sand on the growth of flea larvae at temperature* 23° C. *and relative humidity* 80 *per cent*

Food	Newly hatched larvae used	Larvae which reached adult stage	Time taken to become adults in days	Percentage of reared adults	Remarks
Red corpuscles (3 parts), serum (3 parts), fibrin (1 part) and yeast (7 parts)	20	20	25	100	Average larval life 14 days
	20	19	34	95	
	20	18	25	90	
	20	18	28	90	
Red corpuscles (1 part), serum (1 part) and yeast (2 parts)	20	20	25	100	Average larval life 14 days
	25	22	29	88	
	20	16	25	80	
	22	19	32	86	
Serum (3 parts), fibrin (1 part) and yeast (4 parts)	20	2	54	10	5–10 larvae in each experiment died after about 48 days; minimum larval life 23 days
	20	2	37	10	
	22	4	54	18	
Red corpuscles (2 parts), fibrin (1 part) and yeast (3 parts)	20	6	39	30	Average larval life 15 days
	22	7	32	32	
	22	11	36	50	
Haemoglobin, serum and yeast in equal parts	20	19	27	95	Average larval life 15 days
	20	18	28	90	
	20	20	31	100	
	24	18	37	75	

In order to find out the nutritional value of the different constituents of blood, different combinations were made and the corresponding changes in the rates of growth of the flea larvae were noted. Red corpuscles, serum, fibrin and yeast in proportions noted in Table IV were given as food to flea larvae and 90–100 per cent success in breeding was obtained and the time taken by larvae to become adults was 25–34 days. The results obtained from these experiments agree with those obtained from experiments with blood and yeast as a food. It was further found that red corpuscles, serum and yeast have the same nutritional value as blood or its three constituents combined

with yeast. The combination of fibrin either with serum or with red corpuscles did not affect their respective nutritional value in combination with yeast. The percentage of lagging larvae was considerably higher amongst those that were fed upon serum, fibrin and yeast than those on serum and yeast. It is, therefore, a further proof that fibrin has little nutritional value for the flea larvae, but on the other hand when combined with serum and yeast it has a definitely retarding effect on the rate of growth of the larvae. It is, however, reasonable to assume that red corpuscles and serum form important factors in the nutritional requirements of the flea larvae.

The addition of haemoglobin to serum and yeast gave as good results as blood and yeast. It is, therefore, clear that in the blood the haemoglobin and the serum contain all the parts which are necessary for the normal and successful development of the flea larva. As pointed out by Bacot (1914, p. 516) "The best food gives the quicker development as well as the lowest mortality". As pointed out before, if the red corpuscles and serum are given as food separately with yeast to flea larvae, the mortality is about 50 per cent and the development is delayed. Evidently red corpuscles and serum separately cannot fulfil the nutritional requirements of the flea larvae.

Table V. *Showing the effect of serum with ferrous ammonium sulphate* [$FeSO_4(NH_4)_2SO_4.6H_2O$] *and yeast in equal parts mixed with acid-washed sand on the growth of flea larvae at temperature* 23° C. *and relative humidity* 80 *per cent*

Newly hatched larvae used	Larvae which reached adult stage	Time taken to become adults in days	Percentage of reared adults	Remarks
20	20	33	100	Minimum and maximum larval life 13 and 22 days but in the last two experiments one larva in each lived more than 36 days
20	20	34	100	
20	19	37	95	
20	19	43	95	

It was found that if ferrous ammonium sulphate [$FeSO_4(NH_4)_2SO_4.6H_2O$] is added to the serum exactly in the same concentration as iron in the haemoglobin and yeast mixture and given to the flea larvae as a food (Table V), the percentage of successful breeding was 95–100 per cent, which is the same as with blood mixed with yeast, red corpuscles, serum and fibrin mixed with yeast and red corpuscles and serum mixed with yeast. The percentage of lagging larvae was reduced to minimum. It is, therefore, evident that serum possesses all the proteins necessary for the normal development of flea larvae and that lack of sufficient quantity of iron in it increases the percentage of mortality and prolongs the development. These experiments further prove that the necessity of blood for the normal development of the flea larvae is also due to the fact that flea larvae require blood for their development in order to obtain the necessary supply of iron for their normal growth.

Table VI. *Showing the effect of blood or red corpuscles when mixed with autoclaved yeast and acid-washed sand on the growth of flea larvae at temperature* 23° C. *and relative humidity* 80 *per cent*

Food	Newly hatched larvae used	Larvae which reached adult stage	Time taken to become adults in days	Percentage of reared adults
Blood and autoclaved yeast in equal parts	20	20	30	100
	20	19	33	95
	20	19	31	95
	20	19	36	95
Red corpuscles and autoclaved yeast in equal parts	25	14	37	56
	20	8	40	40
	20	8	37	40

The addition of autoclaved yeast to the blood and red corpuscles (Table VI) gave practically the same percentage of successful reared adults, as is obtained from these when combined with non-autoclaved yeast. These experiments show that as regards the nutritional requirements of the flea larva, the non-autoclaved and autoclaved yeast have the same nutritional value, while, on the other hand, autoclaving kills certain enzymes and stops fermentation by the yeast. Thus, autoclaving affects considerably the constitution of the yeast, and the yeast in my experiments has the same role as other organic substances present in the bed of the host of the fleas.

Table VII. *Showing the effect of autoclaved blood or its constituents mixed with yeast and acid-washed sand on the growth of flea larvae at temperature* 23° C. *and relative humidity* 80 *per cent*

Food	Newly hatched larvae used	Time within which all larvae died in days	Percentage of reared adults	Remarks
Autoclaved blood and autoclaved yeast in equal parts	80	12–16	0	All died in I stage
Autoclaved blood and non-autoclaved yeast in equal parts	72	13–21	0	Most died in I stage, but 12% reached II stage
Autoclaved red corpuscles and autoclaved yeast in equal parts	82	15–17	0	All died in I stage
Autoclaved red corpuscles and non-autoclaved yeast in equal parts	77	11–16	0	All died in I stage
Autoclaved red corpuscles (1 part), autoclaved serum (1 part) and autoclaved yeast (2 parts)	60	20–26	0	Most died in I stage, and 3% reached II stage
Autoclaved red corpuscles (1 part), autoclaved serum (1 part) and non-autoclaved yeast (2 parts)	60	19–25	0	Most died in I stage, and 3% reached II stage

The flea larvae when fed on autoclaved blood either mixed with non-autoclaved yeast or with autoclaved yeast all died without reaching the adult stage. The same thing happened when larvae were fed upon the autoclaved red corpuscles either mixed with non-autoclaved or autoclaved yeast. Most of them died in the first stage. Similar results were obtained when the larvae were fed upon a mixture of autoclaved red corpuscles and autoclaved serum either with non-autoclaved or autoclaved yeast.

Table VIII. *Showing the effect of mixtures of autoclaved or non-autoclaved red corpuscles and non-autoclaved or autoclaved serum mixed respectively with yeast and acid-washed sand on the growth of flea larvae at temperature* 23° C. *and relative humidity* 80 *per cent*

Food	Newly hatched larvae used	Larvae which reached adult stage	Time taken to become adults in days	Percentage of reared adults	Remarks
Autoclaved red corpuscles (1 part), non-autoclaved serum (1 part), and non-autoclaved yeast (2 parts)	20	6	44	30	A few larvae lived more than 37 days
	20	6	43	30	1 larva lived more than 36 days
	20	4	48	20	4 larvae lived more than 48 days
Non-autoclaved red corpuscles (1 part), autoclaved serum (1 part) and autoclaved yeast (2 parts)	20	18	32	90	Average larval life 18 days
	20	16	32	80	
	20	17	31	85	
	20	15	37	75	

When fed upon a mixture of autoclaved red corpuscles, non-autoclaved serum and non-autoclaved yeast, the percentage of the larvae which reached the adult stage was the same as when fed on serum and yeast alone. There were a few lagging larvae. The colour of some of the larvae showed that they had fed only on serum and in a very few cases the larvae showed slight traces of red corpuscles in their alimentary canal.

On the other hand, when fed on a mixture of non-autoclaved red corpuscle, autoclaved serum and autoclaved yeast (Table VIII), 75–90 per cent larvae reached the adult stage. These results are as good as when fed upon blood and yeast. It appears that the native proteins of the red corpuscles which are rich in iron and the denatured proteins of the serum when mixed with yeast have the same food value as blood with yeast.

VI. Summary

A brief summary of previous work done on the life history and the biology of fleas is given. The duration of various well-defined periods during the course of metamorphosis of the flea at temperature 23° C. and 90 per cent relative humidity is recorded.

It is experimentally proved that blood, although the most important part of the nutritional requirements of the flea larvae, is by itself not sufficient for

their normal and successful development. The flea larvae require an additional food which in nature is supplied by the organic refuse present in the bed of the host of the adult fleas.

Experiments were carried out to determine the nutritional value of different constituents of the blood and it was found that the fibrin has no nutritive value to the flea larvae, while the serum and red corpuscles have equal nutritive value. Lagging larvae are the result of some deficiency in the food. The serum possesses all the essential proteins for the normal development of flea larvae but lack of the required amount of iron in it increases the percentage of mortality and delays the development. On the other hand the red corpuscles or haemoglobin, though rich in proteins containing iron but on account of the poverty of some other proteins necessary for the normal development prolong the development and increase the percentage of mortality. It was further experimentally proved that flea larvae require blood for their development in order to obtain the necessary supply of iron for their normal growth. The denatured proteins of the blood and its constituents have no nutritive value for the flea larvae.

I am grateful to Prof. D. Keilin for the suggestion of this problem and for the constant help which he has given me during the progress of this work. I take this opportunity of acknowledging my indebtedness to Prof. P. A. Buxton and Mr H. S. Leeson, both of the London School of Hygiene and Tropical Medicine, for the help given to me on different occasions. I am greatly indebted to Dr P. Tate for revising my manuscript.

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ON *ADELINA TRIBOLII*, A COCCIDIAN PARASITE OF *TRIBOLIUM FERRUGINEUM* F.

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(With Plates XII and XIII, containing Figs. 1–26, and Figs. I–XIX in the Text.)

Introduction

The coccidian dealt with in this paper is a parasite of the blood, fat bodies and connective tissue of *Tribolium ferrugineum*. W. A. Riley and L. Krogh were the first and perhaps the only ones who noticed stages of this parasite, when studying the histology of the larvae of this beetle. They found the pupae and adults uninfected, but in the course of the present work some of them were heavily infected, though only a small number could be examined.

To get fresh larvae a bakery on Trumpington Street proved the most suitable. Some larvae were collected by Prof. D. Keilin several years ago, the progeny of which were still living, and Miss M. Vincent very kindly gave them to me. Old flour from this material was very useful to insure infection in fresh larvae.

Mode of infection

Previous work on the subject throws no light on the method by which cysts are conveyed from the coelom of an infected host to a fresh one. Sixty moulted skins of infected larvae and twenty-five pupal skins were examined and no trace of cysts seen.

It appears that the transmission of the parasite to a healthy larva is effected by the actual ingestion of fragments of infected dead and dry larvae. Flour contaminated with such fragments had a greater number of infected larvae than one which had been sieved through coarse muslin.

How exactly the parasite reaches the coelom is not clear. So far it has not been seen in any part of the gut, which is the most probable channel.

Heavily infected larvae are slower in movement and their tissues more frail, all parts of their body cavity being invaded by the parasite. The nervous system, alimentary canal and malpighian tubules remain uninfected. The parasite is motile in its trophic stages. Many pupae could not be spared, and of the eight examined two were found infected with oocysts and zygotes. Four living adults were examined and found uninfected. Dead and dry adults were then collected from the flour and kept in warm water. The abdominal cavity of 32 per cent was packed with oocysts. Freshly hatched larvae were uninfected.

The percentage of infected larvae in different instars was as follows: first instar = 0 per cent; second instar = 0 per cent; third instar = 13 per cent; fourth instar = 20 per cent; fifth instar = 54 per cent; sixth instar = 63 per cent.

Material and methods

The parasite was studied in living preparations in addition to fixed ones. Living larvae were teased out in distilled water or Ringer's solution, and covered with a cover-slip, the edges of which were sealed with paraffin wax. Under these conditions the parasite remained alive up to 24 hours, which was shown by the fact that merozoites showed movements up to that time. Slight pressure applied to the cover-slip enabled the number of sporocysts to be accurately counted. Increased pressure ruptured the walls of the oocysts and sporocysts, liberating the sporozoites. This is the only means by which living sporozoites can be seen clearly.

Before putting a larva in a fixative, the head and tail ends were quickly cut off and examined for the presence of the coccidian.

The fixatives used were those of Duboscq, Bouin, Carnoy and corrosive alcohol with acetic acid. The last one was found to be the best, especially for Feulgen's reaction. Duboscq's and Bouin's were quite satisfactory for smears and sections to be stained with haemalum and Mann's stain.

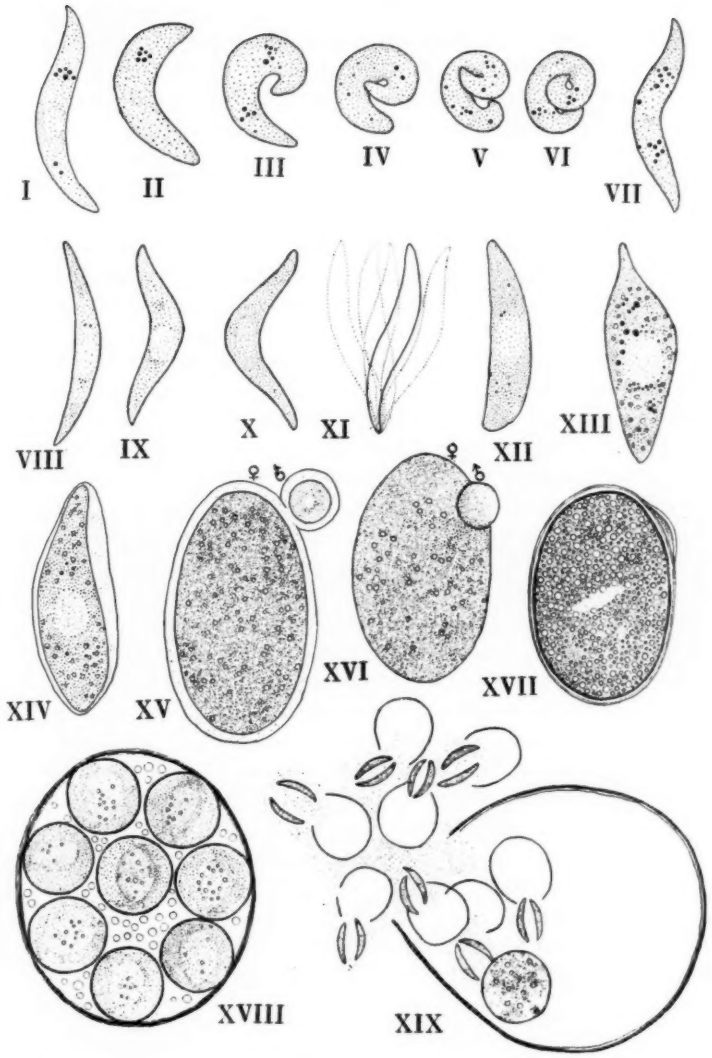
For Feulgen's reaction the fuschin and washing solutions were prepared and used as recommended by Robertson (1927) with the following timings and precautions. Sections were hydrolysed in *N* HCl at 60° C. for 5 min., kept in fuschin solution for 3 hours and counterstained in 2·5 per cent aqueous solution of light green for ½ min. As the sections have a tendency to wash off, the cover-slip should be kept absolutely stationary when in HCl at 60° C. and cooled for a second or two by waving in air before transferring to cold distilled water.[1]

Schizogony

That the parasite begins multiplying by an asexual process is apparent from the fact that the majority of stages seen in mildly infected larvae in early instars are merozoites and schizonts. The newly developed merozoites (Figs. I–X and Fig. 1) are small vermicules about 15μ long and 3μ broad, blunt at both ends with an elongated nucleus, which is usually central and occupies the whole breadth of the organism and about one-fifth of its length. The nuclear membrane is distinct; in its inner surface are deposited chromatin granules which are also scattered throughout the space enclosed. The nucleolus appears as a small chromatic granule only at a slightly later stage (Fig. 2).

The living merozoites (Figs. I–X) are transparent and are provided with a few darker particles in their cytoplasm which are probably reserve material. The nucleus is faintly seen as a lighter area. The organism is capable of three

[1] Feulgen's reaction stains only chromatin material which appears violet red. The nucleolus is stained light green by the counterstain. In Mann's stain chromatin is blue and nucleolus red.



Figs. I–X. Merozoites showing coiling and uncoiling movements. ×3066.
Fig. XI. The spiral movement of a merozoite indicated by dotted lines. ×3066.
Fig. XII. Early stage of a schizont. ×3066.
Fig. XIII. Early stage of a macrogametocyte with a finger-like process. ×3066.
Fig. XIV. Macrogametocyte with gelatinous covering. ×2347.
Figs. XV and XVI. Macro- and microgametocytes. ×2347.
Fig. XVII. Zygote. ×2347.
Fig. XVIII. Oocyst pressed. ×1066.
Fig. XIX. Sporozoites coming out as a result of increased pressure, one of the sporocysts remaining unbroken. ×1066.

kinds of movements: (1) a gliding movement in a straight line along the longitudinal axis of the body without any contraction or expansion; (2) coiling and uncoiling of the body, the uncoiling being performed with a jerk (Figs. I–X); and (3) a spiral movement, in an anti-clockwise direction, of one end of the body, the other end being fixed (Fig. XI).

There is no morphological difference between the two ends of a merozoite. The end which is directed forward in the straight movement and bends first in the coiling and uncoiling is the same in both cases and can be regarded as anterior. The spiral movement of the merozoite, with one end fixed to the slide, perhaps demonstrates the way in which the parasite bores its way into the host tissues. Whether the fixed end is the same as the one regarded as anterior in the first two types of movements could not be seen.

The merozoite grows in size and becomes more or less sausage-shaped (Fig. 3). The nucleus becomes circular and the chromatin granules smaller in size arrange themselves in a ring. The nucleolus is now a large round structure in the centre of the nucleus and is separated from the chromatin by a clear space. When the merozoites enter a cell of the fat body or connective tissue, they become stationary and continue to grow at the expense of the host cell and become oval or spherical in shape. As growth goes on first the nuclear membrane and then the nucleolus disappear (Fig. 4). The chromatin granules increase in number and become arranged in round areas, which are of varying sizes and have a cloudy appearance (Fig. 5). Chromatin granules from these areas condense to form larger granules arranged in smaller groups. These come to lie near the surface of the schizont and are the daughter nuclei (Fig. 6). The cytoplasm now splits round each of these nuclei (Fig. 7). To complete the formation of merozoites the nucleus as well as the body elongates and a nuclear membrane appears round each nucleus. The whole schizont is now a bunch of merozoites, closely applied to each other along their longitudinal axes (Fig. 8). These merozoites gradually become separated from each other; begin to move about and repeat the cycle again or develop into gametocytes. The number of merozoites produced varies with the size of the schizont. The length of the schizont varies from 15·5 to 30·0μ and the breadth from 6·25 to 20·0μ. The number of merozoites produced by them is 8–26. The appearance of the cytoplasm of schizonts is similar to that of the merozoites.[1]

Sporogony

For the process of sexual reproduction some of the merozoites grow much larger, passing first through a stage which has a finger-like process at one end, capable of protuding and retracting and moving from side to side (Fig. XIII). These give rise to macrogametocytes. The cytoplasm of these has a large number

[1] In *Adelina octospora* Hesse describes two kinds of schizogonies, one undifferentiated and the other leading to the formation of micro- and macrogametocytes. No such dimorphism has been seen in *Adelina tribolii*.

of sphaerules in addition to food particles. The process gradually disappears and the organism secretes a gelatinous covering round itself (Figs. XIV, XV). Fully developed macrogametocytes (20·8–48·6μ by 15·6–32·5μ) are oval in shape, stationary and covered with a gelatinous substance. Their cytoplasm is yellowish brown, opaque and rich in refringent bodies. Stationary stages are usually found inside the host cells, but may be present in the coelomic cavity. In fixed and stained preparations the finger-like process in the early stages of the females and the gelatinous covering are not seen, and the cytoplasm has an alveolar structure (Fig. 11). The nucleus is similar to that of the corresponding sausage-shaped stage of the schizogonous cycle, already described, except that the chromatin ring is broader on one side. In later stages there are always one or two darkly staining large-sized granules present in the chromatin (Figs. 13, 15, 16, 19). It is shown by Mann's stain and Feulgen's reaction that these granules are a part of the chromatin material.

The merozoites giving rise to microgametocytes become rounded off on approaching a macrogametocyte without undergoing any structural change (Figs. 10, 13). They become covered by the gelatinous substance by means of which they adhere to the macrogametocyte (Fig. XV). After some time the gelatinous covering gradually disappears and the male becomes partly embedded in the body of the female (Fig. XVI and Figs. 14, 15). More than one microgametocyte may be found attached to the macrogametocyte; as many as six have been seen (Fig. 13). Although division of the nucleus may commence in all the attached males, only one produces microgametes. The diameter of microgametocytes varies from 7·8 to 15·0μ.

FERTILIZATION

The cytoplasm of macrogametocytes shows deeply staining masses which are most numerous a little before or at the time of its union with the microgametocyte (Fig. 14). With Feulgen's reaction they were not stained (Fig. 12). Before fertilization these masses seem to move outwards and come to lie for some time near the periphery of the macrogametocyte (Fig. 15) and ultimately disappear.

In the microgametocyte first the nuclear membrane and later the nucleolus disappear (Fig. 15). The chromatin granules first divide into two groups, each one of which divides again to form four daughter nuclei (Figs. 16, 17, 18). These nuclei develop into four microgametes, which are darkly staining, structureless and more or less comma-shaped bodies. They come out through that part of the microgametocyte which is in contact with the macrogametocyte, and travel over the surface of the latter. In the meantime the nucleus of the macrogametocyte moves towards the opposite pole (Fig. 19). One of the microgametes enters the macrogametocyte at this pole and unites with its nucleus, and a synkarion is formed. The zygote secretes round itself a cyst wall, made up of several layers of yellowish brown material. The remains of the

microgametocyte can be seen attached to the zygote for some time (Fig. XVII). After fertilization the nucleus of the zygote becomes more or less spindle-shaped, and shows strands and granules of chromatin (Fig. 20). The cytoplasm in the neighbourhood of the spindle is finely granular in structure. The spindle simply stretches and breaks in an irregular manner giving rise to 4–5 masses of chromatin granules, some of which divide again (Figs. 21, 22). Within the cyst wall a closely applied membrane gradually appears round the zygote (Figs. 23, 24). The cytoplasm of the zygote begins to divide by a process of budding into sporoblasts (Fig. 23). A firm cyst wall forms round each sporoblast, the nucleus of which divides to give rise to two sporozoites, thus forming sporocysts. The sporocysts are spherical, 10·4μ in diameter, and contain a fluid in addition to two sporozoites (Figs. XVIII, XIX). The sporozoites lie along the wall of the cysts, their concave sides facing each other, and their pointed ends sometimes overlapping (Fig. 26). A fluid containing prominent globules is also present outside the sporocysts in an oocyst. When sporoblasts are formed, the membrane which enveloped the zygote and was at first closely applied to the inner surface of the cysts, becomes detached at both poles (Fig. 24).

Most of the oocysts are oval, but some are absolutely spherical. The cyst wall is strong and elastic. In length the oocysts vary from 26·0 to 50·0μ and in width from 22·5 to 36·4μ, and the number of sporocysts in an oocyst varies from 2 to 14, 8 and 12 being more common. A sporozoite is 9μ long and 1·12–2·0μ broad, and its nucleus is terminal (Fig. 26).

Systematic position of *Adelina tribolii*

There is great similarity in the method of reproduction of the species of the genus *Adelea A. Schneider* 1875 and *Adelina Hesse* 1910. Hesse reserved the former for the species *Adelea ovata*, and created the latter on discovering *Adelina octospora*, giving the characteristics for distinguishing the two genera, which can be summed up as follows:

Adelea: oocysts ovoid with very weak walls, moulded along the walls of the sporocysts. Sporocysts discoidal, bivalved, with thin non-resisting wall. Number of sporocysts in an oocyst often more than a hundred.

Adelina: oocysts spherical or subspherical with firm wall. Sporocysts also spherical with firm wall. Lesser number of sporocysts in an oocyst, viz. frequently 4–8 and exceptionally 12–20 in coelomic types.

To me it appears that the spherical or discoidal bivalved nature of the sporocysts is the most reliable of all the characters for differentiating the two genera. From A. Schneider's definition of the genus *Adelea* it is gathered that oocysts may be spherical or ovoid.

According to the figures of Schellack & Reichenow (1915), who have done a great deal of work on *Adelea ovata* Schneider, the wall of the oocyst does not seem weak or moulded along the walls of the sporocysts. The number of sporocysts in an oocyst as given by them is 16–32, which is a high figure but not as high as that given by Hesse.

Following Hesse's line of classification I place *Adelina tribolii* in the genus *Adelina*, in spite of the fact that oocysts are both ovoid and spherical. Hence ovoid and spherical oocysts become common to the two genera. W. A. Riley & L. Krogh (1927) thought that the parasite was closely related to *A. mesnili* Perèz. *A. mesnili* was briefly described, without any illustrations, by Perèz in 1899. From his description it appears that though there are some resemblances in certain stages, they can in no way be regarded as one species. The following characters serve to differentiate the two:

In *A. tribolii* the number of sporocysts in an oocyst varies from 2 to 14, 8 and 12 being more common. The diameter of the sporocysts is 10·4μ. The number of sporocysts in an oocyst of *A. mesnili* is between 6 and 8, rarely increasing to 9, and decreasing to 4 and even 3; the diameter of the sporocyst is 15μ. The number of merozoites produced by the schizonts is 8–26 in *A. tribolii*, while in *A. mesnili* the number is very variable, but is generally 20–30. The size of the macrogametocyte of *A. tribolii* is smaller than that of *A. mesnili*, viz. length 20·8–48·6μ and breadth 15·6–32·5μ in the former, and length 30·0–50·0μ and breadth 20·0–35·0μ in the latter. The macrogametocyte also has a small finger-like process in its early stages, which is unknown in *A. mesnili*. There is a membrane inside the cyst wall also unknown in *A. mesnili*. *A. tribolii* is in my opinion more closely related to *A. tenebrionis* Sautet. The variation in the number of sporocysts in an oocyst is 2–14 in *A. tribolii* and 2–12 in *A. tenebrionis*, their sizes being 10·4μ and 10·0–12·0μ respectively. In *A. tribolii* the oocyst is 26·0–50·0μ in length and 22·5–36·4μ in breadth, and in *A. tenebrionis* the diameter of the oocyst is 20·0–35·0μ. The number of merozoites produced by a single schizont of *A. tribolii* is 8–26, while that of *A. tenebrionis* is 8–10. *Tenebrio molitor* larvae kept for more than 3 months in flour contaminated with both living and dead infected larvae of *Trilobium ferrugineum* remained uninfected. This non-infection combined with other differences keeps the two species distinct. Therefore I regard the *Adelina* species of *Trilobium ferrugineum* F. as a new one and call it *Adelina tribolii*.

EXPLANATION OF PLATES XII–XIII

PLATE XII

Fig. 1. Newly developed merozoite. ×2472.
Fig. 2. Later stage of the merozoite showing a small nucleolus. ×2472.
Fig. 3. Sausage-shaped stage with large round nucleus, having large nucleolus and chromatin arranged in a ring. ×2472.
Fig. 4. Schizont in which the nuclear membrane has disappeared. ×2472.
Fig. 5. Schizont with groups of fine chromatin granules. ×2472.
Fig. 6. Schizont inside the host's tissue with darkly staining nuclei having large-sized chromatin granules. ×2472.
Fig. 7. Cytoplasm splitting round the nuclei of the schizont. ×2472.
Fig. 8. Schizont completely transformed into a group of merozoites. ×2076.
Fig. 9. A group of merozoites inside the host tissue cut transversely. ×2307.
Fig. 10. Merozoite giving rise to macro- and microgametocytes. ×2472.
Fig. 11. Early stage of macrogametocyte. ×2347.

Fig. 12. Micro- and macrogametocytes treated with Feulgen's reaction, chromatin stained violet red and nucleolus lightly stained by the counterstain. ×2347.

Fig. 13. Macrogametocyte with six microgametocytes attached to it. ×2347.

Fig. 14. Macro- and microgametocytes inside the host cell stained with Mann's stain, showing darkly staining bodies present in the cytoplasm of the female, and nucleolus stained red. ×2347.

Fig. 15. Macro- and microgametocytes stained with Mann's stain, showing darkly staining masses near the periphery and nuclear membrane disappeared from the nucleus of the male gametocyte. ×2347.

Fig. 16. Male and female gametocyte, showing the development of microgametes in the former. ×2000.

PLATE XIII

Figs. 17 and 18. Showing the divisions of the nucleus of the microgametocyte. ×2347.

Fig. 19. Four microgametes have come out of the microgametocyte and one is shown entering the female gametocyte. ×2347.

Fig. 20. Stage showing synkarion. ×2347.

Fig. 21. Zygote with synkarion in the act of division. ×2347.

Fig. 22. Formation of daughter nuclei in the zygote. ×2347.

Fig. 23. Oocyst containing sporoblasts. ×2347.

Fig. 24. Fresh oocyst with eight sporocysts. ×1244.

Fig. 25. Fresh oocysts with four sporocysts. ×1244.

Fig. 26. Sporozoites inside a sporocyst. ×2500.

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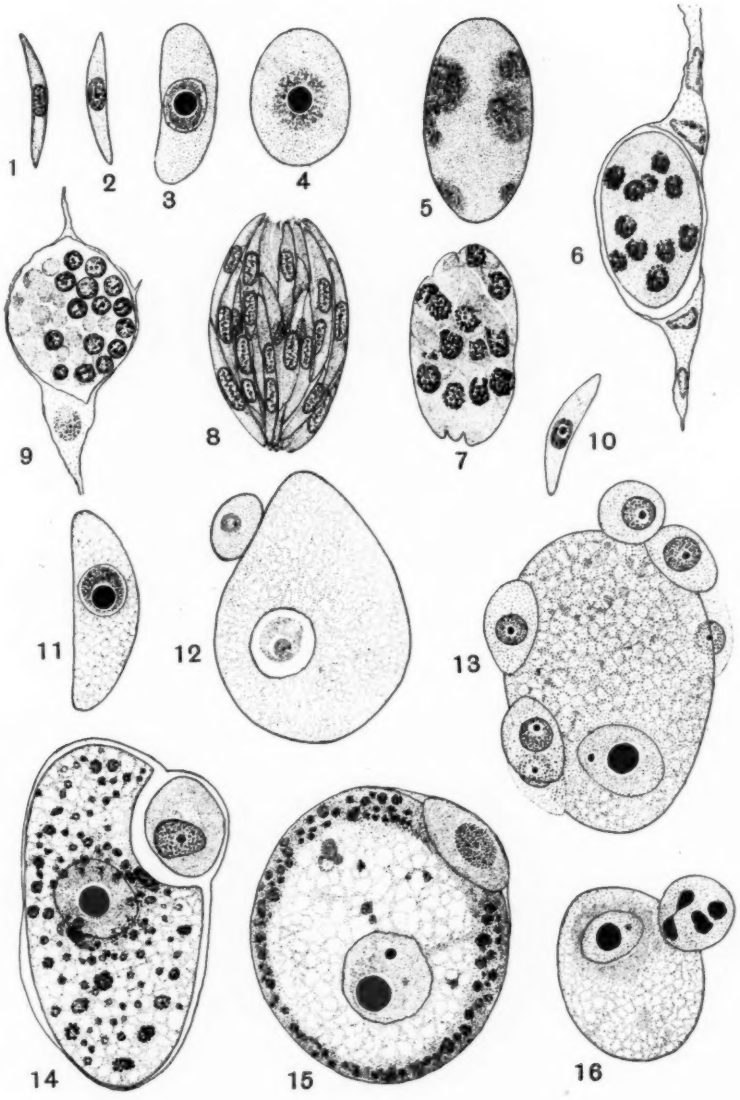

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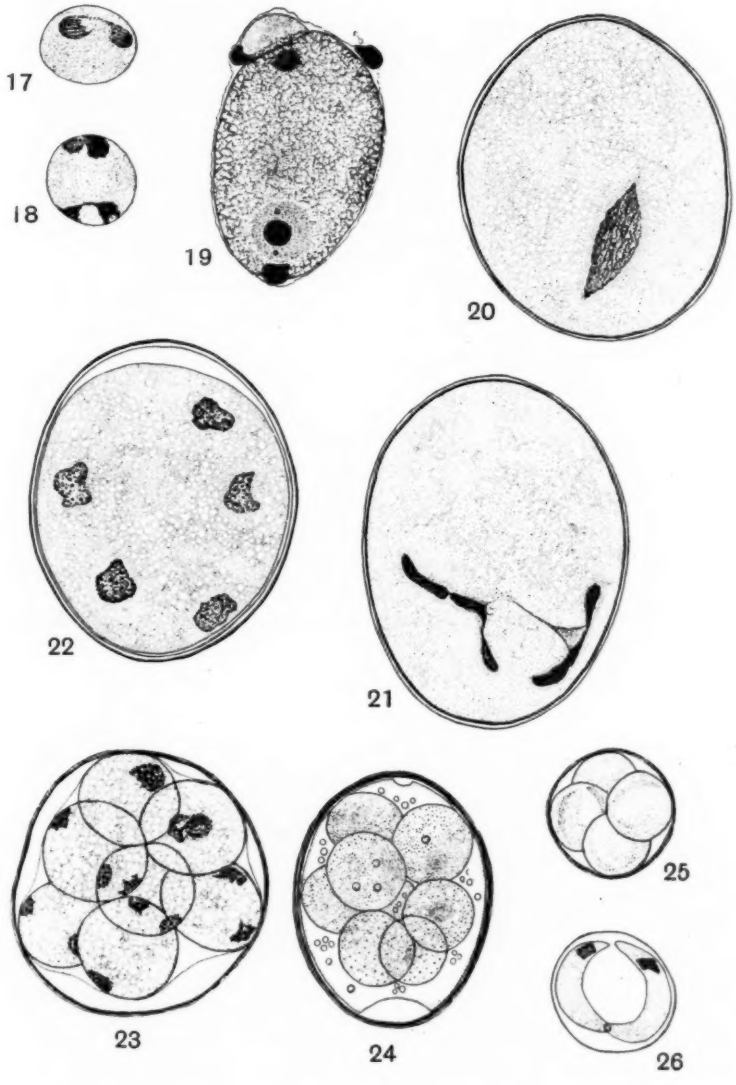

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A COMPARATIVE ACCOUNT OF THE LARVAE OF *TRICHOMYIA URBICA* CURTIS, *PSYCHODES ALBIPENNIS* ZETT., AND *PHLEBOTOMUS ARGENTIPES* ANN. & BRUN. (DIPTERA: PSYCHODIDAE)

By D. KEILIN and P. TATE

From the Molteno Institute, University of Cambridge

(With 45 Figures in the Text)

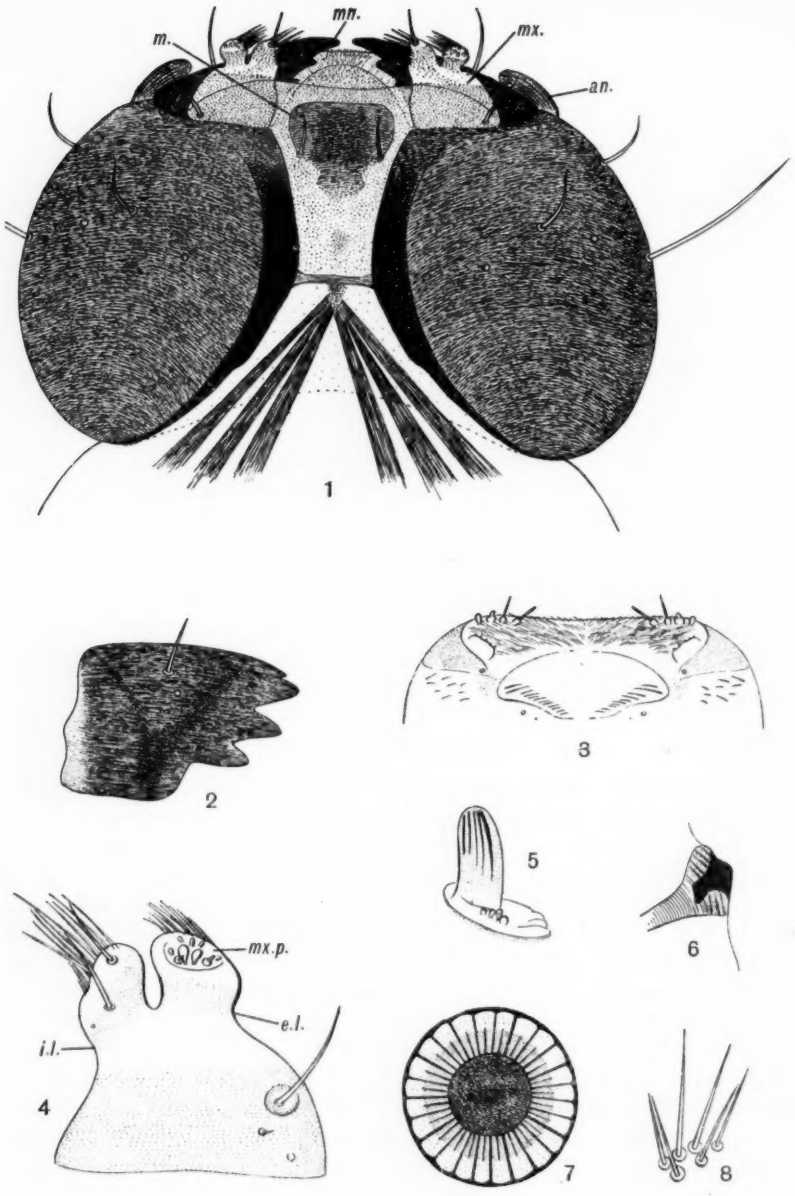
Previously one of us (Keilin, 1914) described very briefly in a preliminary note the larva of *Trichomyia urbica* Curtis and showed that, while the larvae of the other genera of Psychodidae are all either aquatic or semi-aquatic, the larva of *Trichomyia* is a wood-borer and lives in galleries which it makes in rotten wood. In correlation with its mode of life it exhibits interesting morphological features in which it differs greatly from other psychodid larvae. In the present paper a fuller description of the external morphology of the larva of *Trichomyia urbica* will be given and it will be compared with the larvae of two other genera of psychodids, *Psychodes* and *Phlebotomus*.

Trichomyia urbica Curtis

The larvae have been collected in the rotten wood of fallen trees in which they make galleries running in the direction of the grain. The larvae make the galleries by eating the wood in the same manner as other xylophagous larvae. The fully grown larvae are about 0·5 cm. long and are cylindrical throughout their length and do not taper anteriorly or posteriorly. The body consists of a head, three thoracic and eight abdominal segments. The segmentation is obscured in the last seven abdominal segments by the presence of folds near the anterior margin. Each of the segments two to seven bears a raised locomotory pad on the ventral surface near the anterior margin. The eighth segment has a continuous fold near the middle and appears to be two segments, the posterior of which bears the terminal anus (Fig. 13, *A*.) while the anterior part bears the posterior spiracles on the dorso-lateral region (Figs. 9, 10, 13, *p.sp.*). The first thoracic segment bears the anterior spiracles (Figs. 9, 10, *a.sp.*).

The body, unlike that of other psychodid larvae, is devoid of long branched or plumose setae and is only finely pilose with a number of small bristles on each segment. The pilosity of the body and the arrangement of the small bristles is shown in Figs. 11, 12.

The head of the larva is large and globular and is very strongly chitinized (Fig. 1). The antennae are strongly chitinized and are closely applied to the



Figs. 1–8. *Trichomyia urbica* Curtis. 1, head, ventral; 2, mandible, external; 3, labrum, ventral; 4, maxilla, external; 5, antenna; 6, anterior spiracle, optical section; 7, anterior spiracle, surface view; 8, setae representing the sensory vestiges of the thoracic legs. *an.* antenna; *e.l.* external lobe of maxilla; *i.l.* internal lobe of maxilla; *m.* mentum; *mn.* mandible; *mx.* maxilla; *mx.p.* maxillary palp.

head (Fig. 1, *an.*). They consist of an oval base from which arises a stout, slightly curved, digitiform process, near the base of which is a group of five sensory papillae (Fig. 5). The strong mandibles are heavily chitinized and bear four strong teeth on the anterior margin (Fig. 2). They are devoid of the numerous sensory processes and setae possessed by most other psychodid larvae and bear only a single bristle near the middle of the dorso-external margin, and, ventral to it, a sensory pit.

The maxillae are soft plates and are divided into two lobes (Fig. 4). The external lobe (*e.l.*) which represents the maxillary palp bears a terminal bunch of five bristles and a ventro-terminal circular area of thin chitin on which are borne nine sensory processes, including two hairs, five small papillae and two large claviform papillae (Fig. 4, *mx.p.*). The inner lobe (*i.l.*) bears a marginal fringe of setae and has two stout setae and a sensory pit on the internal surface. Basally a single large seta arises from the region representing the cardo, posterior to it there is a minute seta and near the dorso-posterior margin there is a sensory pit.

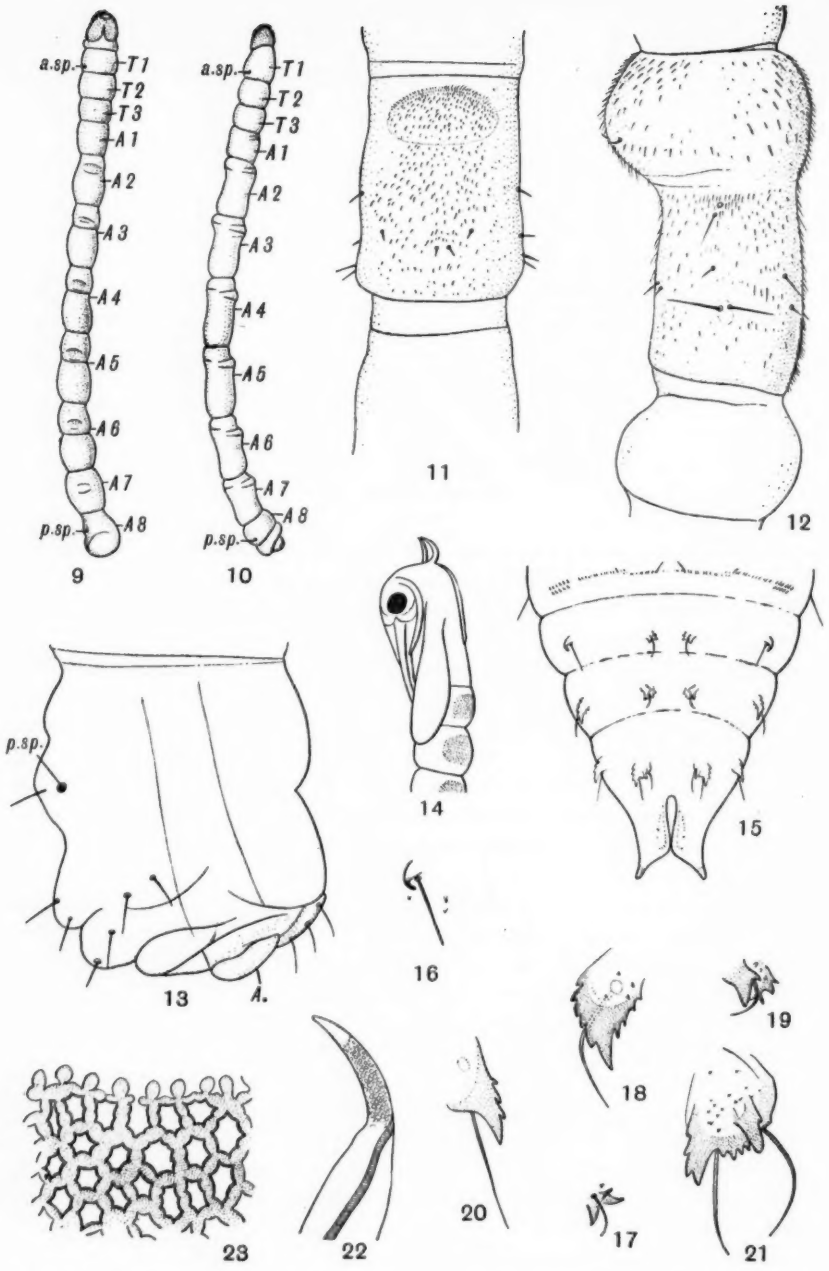
The labrum is broad and fleshy and is supported by lateral bands of chitin (Fig. 3). The anterior margin bears a group of four sensory processes, two setae and two papillae on each side, posterior to which, on each side, there is a sensory pad bearing five minute hairs. These pads probably represent the premandibles. There are sensory pits on the inner terminations of the chitinized bands; and there is a pair of pits posterior to a shield-like chitinized area which probably represents the epipharynx and which lies between and posterior to the chitinized bands.

The lower lip is very weakly developed and only a delicate transverse plate, with a central shield-like more chitinized area, is distinguishable from the ventral surface (Fig. 1). This delicate plate probably represents the mentum (Fig. 1, *m.*).

The spiracles have a central plug from which rods or trabeculae radiate to the rim. Thus the air is admitted through a circle of clefts surrounding the central plug (Figs. 6, 7). The anterior and posterior spiracles resemble each other except that the posterior ones are larger than the anterior.

The sensory vestiges of the thoracic legs are represented by groups of six stout setae (Fig. 8).

The pupa has long anteriorly curved respiratory horns, the apical parts of which are perforated by numerous irregular openings forming cribiform areas (Figs. 14, 22, 23). On the dorsal surface of each of the last three segments there are four characteristically shaped scales arranged in two pairs, median and lateral, and from beneath the base of each scale one or two sensory setae arise (Figs. 15, 16–21).



Figs. 9–23. *Trichomyia urbica*. 9, whole larva, ventral; 10, whole larva seen from right side; 11, abdominal segment, dorsal; 12, abdominal segment seen from left side; 13, eighth, or last, abdominal segment seen from right side; 14, anterior part of pupa, lateral; 15, last three segments of pupa. 16–17, 18–19, 20–21, lateral and median scales and setae of the dorsal surface of the last three segments of the pupa; 22, pupal respiratory horn; 23, part of the perforated area of a pupal respiratory horn. *A.* anus; *a.sp.* anterior spiracle; *p.sp.* posterior spiracle.

Psychodes albipennis Zett.

The larva of *Psychodes albipennis* Zett. may be taken as an example of the genus *Psychodes*. The fully grown larva is about 3·5 mm. long and the body is nearly cylindrical but slightly compressed dorso-ventrally. Anteriorly it narrows towards the head which is large, well chitinized and shaped like a truncated cone. Posteriorly the body narrows sharply from the penultimate segment and the last abdominal segment is prolonged into a slender siphon at the apex of which are the posterior spiracles. There are three thoracic and eight abdominal segments, but the segmentation is obscured by secondary folds and by the dense covering of ciliated scales and branched hairs so that careful examination of the chaetotaxy is necessary to determine the segmentation. On the dorsal surface of segments 5, 6 and 7 there are chitinized plaques, a small single one on segment 5 and three indefinite plaques on each of segments 6 and 7. Laterally each segment, except the first thoracic and the eighth abdominal, bears tubercles from which long branched setae arise. The general appearance of the larva and the chaetotaxy of the dorsal surface is shown in Fig. 24.

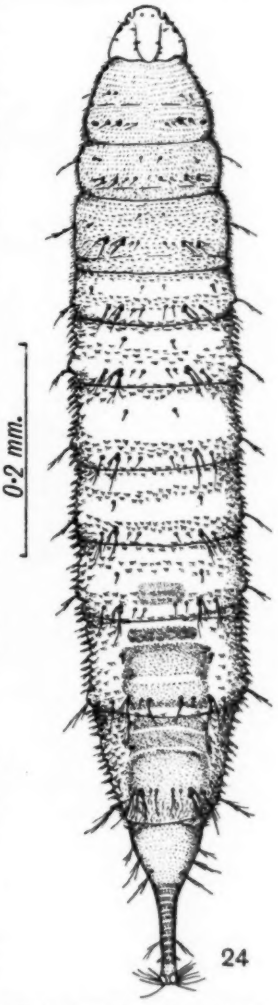


Fig. 24. *Psychodes albipennis* Zett. Whole larva, dorsal.

As already mentioned, the posterior spiracles are borne at the end of the siphon formed by the last abdominal segment. A pair of small anterior spiracles are present on the first thoracic segment.

The sensory vestiges of the thoracic legs (Fig. 29) consist of groups of four setae, one long and three short ["pedichaeten" of Feuerborn (1927)].

The head is strongly chitinized, and has a U-shaped epicranial suture.

The sessile antennae, which are borne on weakly chitinized areas encircled by strongly chitinized arms, are formed of a domed vesicle, shaped like an inverted watch-glass, posterior to which there is a group of sensory processes (Fig. 28). The sensory processes comprise, internally, a clavate process, external to that a group of fine setae, then a group of three clavate processes and, finally, a group of long, fine, setae.

The general appearance and arrangement of the mouth parts is shown in Fig. 25 as seen from the ventral aspect. The labrum is well developed and has numerous long curved spines on the anterior margin and the ventral surface. Epipharynx and premandibles (Fig. 25, *p.md.*) are present and resemble those described in *Maruina indica* by Feuerborn (1933). The labrum of *Psychodes* is very similar to those of *Trichocera*, *Rhyphus* (Keilin, 1912) and *Scatopse*.

The mandibles are complicated structures (Fig. 25, *mn.*; Figs. 26, 27). Each is distinguishable into two parts distally, the dorsal and ventral lobes. In external aspect there is a weakly chitinized dorsal lobe which terminally bears one long pectinate process and one longer plumose process. A strong

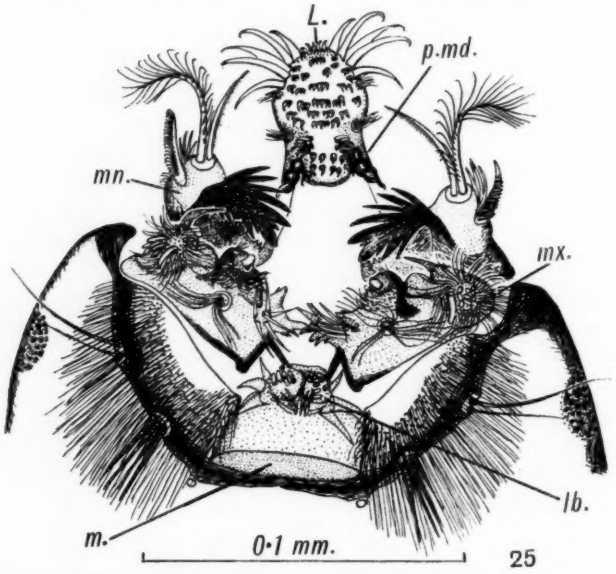
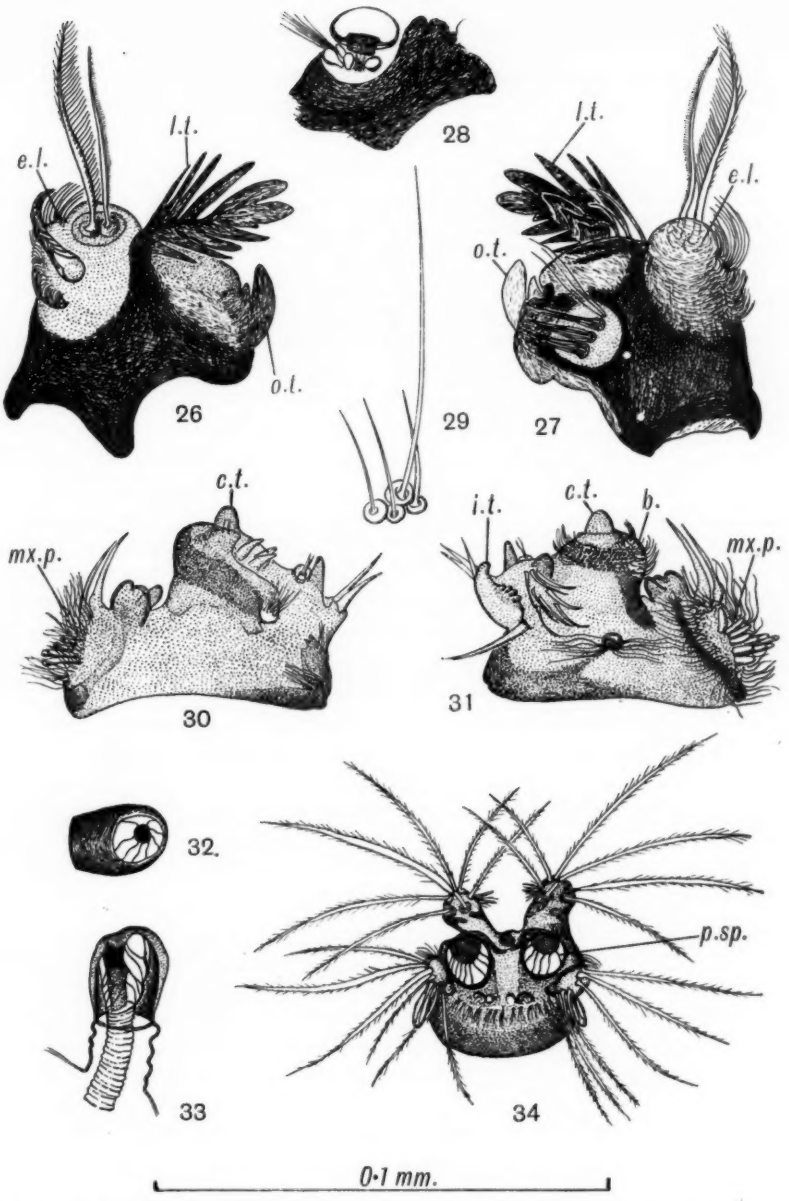


Fig. 25. *Psychodes albipennis*. Head of larva, ventral. The head has been slightly compressed in order to separate the mouth parts. *L.* labrum; *lb.* labium; *p.md.* premandibles. For other letters see Figs. 1–8.

pectinate process arises posteriorly to these terminal processes and runs around the lobe in a dorso-anterior direction. Posterior to this pecten there is an arc of curved hairs. Externally the ventral lobe of the mandible is composed of a large pinnate, leaf-like process or compound tooth, which curves downwards and inwards. Posterior and opposed to it is a large curved tooth with a small supplementary tooth at its base (Figs. 26, 27, *o.t.*). In internal aspect (Fig. 27) the leaf-like process is seen to be composed of three series of teeth, two lateral and one ventral. Near the base of the compound tooth there is a group of three curved hairs. Internal and posterior to the opposed lateral tooth there is an area of thin chitin from which arise four bunches of setae. Near the internal ventral margin there are two sensory pits.



Figs. 26–34. *Psychodes albipennis*. 26, mandible, external; 27, mandible, internal; 28, antenna; 29, setae representing the sensory vestiges of the thoracic legs; 30, maxilla, internal; 31, maxilla, external (in this specimen the compound tooth on the median border is slightly misplaced; normally it is directed so that the convex edge is anterior and the supplementary teeth are on the posterior edge of the main tooth); 32, prothoracic spiracle; 33, prothoracic spiracle in optical section; 34, apex of the siphon with the posterior spiracles (this figure is the same scale as Fig. 25). *b.* boss of maxilla (=mala); *c.t.* conical tooth of maxilla; *i.t.* internal teeth of maxilla; *l.t.* leaf-like compound tooth of mandible; *o.t.* opposed tooth of mandible. For other letters see legends to previous figures.

The maxillae are roughly oblong in shape and bear numerous setae and sensory processes (Fig. 25, *mx.*; Figs. 30, 31). They are articulated so that their long axes are nearly dorso-ventral but inclined inwards towards the mid-ventral line. Two areas may be seen in the maxilla, an outer, or dorsal, area representing the maxillary palp (*mx.p.*) which is delimitated by a ring-like band of chitin and distally bears two long, curved, weakly chitinized teeth, and laterally has a group of about twelve digitiform, curved, sensory processes which are surrounded by a dense ring of long hairs. The inner, or ventral, area has medially on the anterior margin a strongly chitinized boss (*b.*) in the centre of which is a cone-shaped tooth (*c.t.*). The external rim of the boss is thickly covered with short curved hairs. Probably this region represents the mala. The ventro-internal border bears a number of medially directed teeth. Near the middle there is a single long, curved tooth, in front of that there is a large curved tooth with three secondary teeth on its posterior margin, and anteriorly there are three or four long, slender, weakly chitinized teeth (Fig. 31). On the anterior margin, internal to the boss, there are two strong knob-like teeth, and, posterior to them, there is a group of tapering, curved setae. In the centre of the external surface of the body of the maxilla there is a circular area of weak chitin from which a group of long hairs arises. The interior surface of the maxilla has a group of short hairs at the base of the boss, three short tooth-like processes internal to the conical tooth, a small sensory process bearing two hairs on the anterior margin, and two groups of fine hairs, one on the internal margin and one at the internal posterior corner (Fig. 30).

The mentum (Fig. 25, *m.*) is a plain narrow transverse plate which is weakly chitinized. Dorsal to the mentum is the labium (*lb.*) which consists of a fleshy cushion bearing a median ventral group of setae, two lateral raised pads each bearing seven sensory processes, and, on each side, three broad, curved teeth. The hypopharynx is a transverse bar of chitin produced into long lateral arms and lies dorsal to the labium from which it is separated by the opening of the salivary duct.

The posterior spiracles (Fig. 34, *p.sp.*) consist of a central plug or scar which radiating trabeculae connect with the external rim. They are placed at the apex of the siphon which is produced into two long postero-lateral and two short antero-lateral lobes. Each lobe bears six long, plumose radiating setae; and there are a number of short hairs and sensory processes surrounding the spiracles.

The anterior spiracles are smaller than the posterior ones, but also consist of a central plug and radiating trabeculae (Figs. 32, 33).

Phlebotomus argentipes Ann. & Brun.

The body of this larva shows a distinct head, three thoracic and eight abdominal segments, but the segmentation is obscured by secondary folds and careful study of the chaetotaxy is necessary to determine the limits of

the segments. Consequently some authors describe the larva as having thirteen segments (Perfiljew, 1928). The body has a dense coat of small tubercles and each segment has a girdle of long setae which may be brush-like or simple. Ventrally each abdominal segment has a locomotory pad which in the case of the last segment is bifid and functions as pseudopods.

The posterior segment is produced into two dorso-lateral lobes from which spring several very long setae in addition to some short brush-like setae (Fig. 35). The anus (Fig. 35, *A.*) opens terminally, ventral to the setae-bearing lobes, and dorsal to the pseudopods of the eighth abdominal segment. The apparent subdivision of the eighth abdominal segment causes the posterior spiracles to appear to open laterally on the penultimate segment, but they really open slightly anterior to the middle of the eighth, or last, abdominal segment (Fig. 35, *p.sp.*). A pair of prothoracic spiracles are also present.

The head is nearly cylindrical and bears a number of long setae and sensory pits.

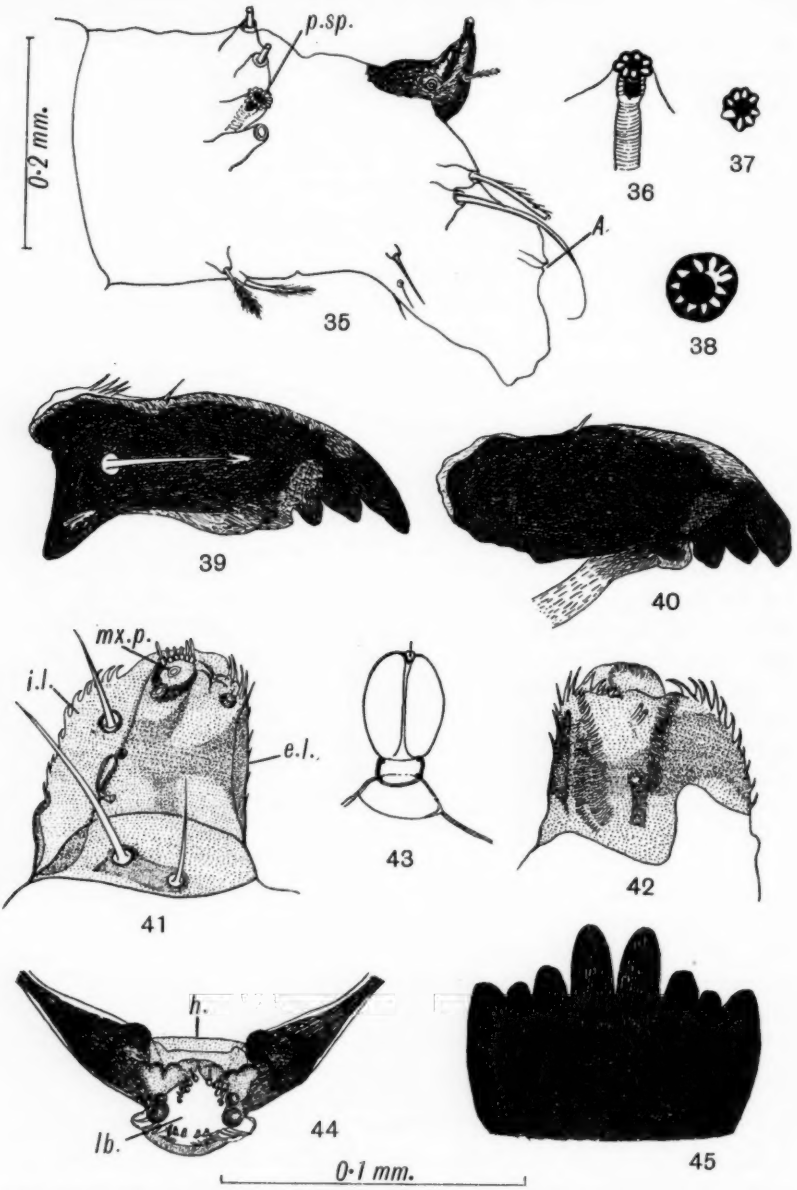
The antennae consist of two very short proximal segments and an inflated third, distal, segment which is constricted by a median longitudinal fold and has a small terminal seta (Fig. 43).

The labrum projects forwards from the clypeus and has a delicate protrusible anterior portion. Ventrally there are ill-defined lateral teeth and a weakly chitinized median area which may represent premandibles and epipharynx respectively.

The mandibles (Figs. 39, 40) are very strongly chitinized, elongated, and curve inwards and downwards. The ventro-distal margin is produced into four angular teeth of which the fourth is only weakly developed. Rudiments of a fifth tooth may be discernible. The external surface bears two setae and a sensory pore, and the proximal part of the dorsal edge bears a few short setae.

The maxillae (Figs. 41, 42) are nearly square delicate plates which are articulated so that they lie in the vertical plane at the sides of the mouth. On the external surface a proximal plate bearing two long setae is demarcated (Fig. 41) and probably represents the cardo of de Meijere (1916). The body of the maxillae is divided by a horizontal fold into a ventro-external region (Fig. 41, *e.l.*) which distally has a circular area of thin chitin bearing two sensory pits and an anterior semicircle of seven sensory processes. This area probably represents the maxillary palp (Fig. 41, *mx.p.*). From the longitudinal fold, near the proximal end, a curved sensory process arises, anterior to which there is a sensory pit. The part of the maxilla dorsal and internal to the fold (Fig. 41, *i.l.*) bears one long seta and has the margin produced into a row of forwardly curved small, weak, teeth. The internal surface of the maxilla bears a pair of digitiform sensory processes, a sensory pit, and several longitudinal bands of short hairs (Fig. 42).

The mentum is a strongly chitinized, curved plate with the anterior margin produced into strong blunt teeth (Fig. 45). There are four teeth on each side



Figs. 35–45. *Phlebotomus argentipes* Ann. & Brun. 35, eighth, or last, abdominal segment seen from the left side, showing the position of the posterior spiracles and of the anus. All the long setae are shown broken off close to the base. 36, anterior spiracle; 37, anterior spiracle, surface view; 38, posterior spiracle, surface view; 39, mandible, external; 40, mandible, internal; 41, maxilla, external; 42, maxilla, internal; 43, antenna (same scale as Figs. 26–33); 44, labium and hypopharynx, anterior aspect; 45, mentum, ventral. *h.* hypopharynx. Other letters as in previous figures.

of the median line of which the inner ones are much larger than the three lateral ones.

The labium (Fig. 44) lies dorsal to the mentum and consists of a delicate chitinous membrane, supported dorsally by the hypopharynx (*h.*), and bearing on each side of the anterior face six dorsal and three ventral sensory processes, three small curved setae laterally and a ventral group of fine hairs.

The posterior spiracles have a central plug and a thick rim from which stout rays extend to the plug producing a rosette-like appearance in surface view (Fig. 38). There are about twelve openings between the rays. The prothoracic spiracles are similar in structure except that they are smaller and have only six to eight openings (Figs. 36, 37).

SUMMARY AND DISCUSSION

From the foregoing brief descriptions it is clear that the larva of *Trichomyia urbica* stands apart from other psychodid larvae not only in its mode of life, as a wood-borer, but also in its morphology. The regularly cylindrical body, devoid of dorsal plaques and long setae and the densely chitinized head give it a very different appearance from that of other psychodid larvae.

The small and heavily chitinized antennae are very different from those found in larvae of *Phlebotomus*, in which they are 3-segmented; *Psychodes*, in which they are delicate and dome-like; *Sycorax*, in which they are long and 4-segmented (Feuerborn, 1933); and *Maruina*, in which they are formed of small rods (Feuerborn, 1933).

The massive toothed mandibles, devoid of sensory processes, differ greatly from those of other genera of psychodids except *Phlebotomus*. *Maruina*, *Sycorax* (see Feuerborn, 1933), and *Psychodes* all have mandibles with large leaf-like processes and many long plumose or pectinate setae and sensory hairs or processes.

The maxillae, in all the three genera examined above, are delicate plates with ill-defined areas representing the maxillary palps, and with many delicate teeth, long setae and sensory processes arising from the body of the maxilla.

The lower lip in *Trichomyia* is very simple and the mentum is hardly distinguishable. In this respect it resembles *Psychodes* where the mentum is a simple transverse plate, and *Maruina* (see Feuerborn, 1933) in which it is similar except that the anterior border of the plate is fringed with long setae. In *Phlebotomus* the mentum is a densely chitinized plate with strong teeth on its anterior margin.

The anterior spiracles appear to be much alike in all the genera in which they are present; but in the position of the posterior spiracles *Trichomyia*, in which they are placed far back on the dorso-lateral region of the last segment, differs from all other genera examined except *Phlebotomus*, in which they occupy a similar position.

Therefore, as regards the shape of the body, nature of the cuticle, absence

of long setae and of dorsal plaques, and the structure of the antennae and labrum, the larva of *Trichomyia* differs from all other psychodid larvae. It shares with the larva of *Phlebotomus* the possession of strong, simple, toothed, mandibles devoid of long setae and sensory processes. It also resembles *Phlebotomus* in the poorly defined epipharynx and premandibles on the labrum, and in the position of the posterior spiracles near the middle of the last segment. The weakly developed mentum of *Trichomyia* larvae, however, differs greatly from that of *Phlebotomus* larvae in which the mentum is very strongly developed as a toothed plate.

ACKNOWLEDGEMENT. We wish to express our thanks to Prof. P. A. Buxton, London School of Hygiene and Tropical Diseases, for supplying us with some larvae of *Phlebotomus*, and to Dr F. W. Edwards, British Museum of Natural History, for identifying the imagoes.

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ACLADIUM CASTELLANII PINOY AND THE EXISTENCE OF THE SO-CALLED ACLADIOSIS OF CASTELLANI

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(With Plate XIV and 9 Text-figures)

In October 1916, A. Castellani(1) gave an account in the *British Medical Journal* of a disease which he had observed since 1907 in Ceylon and of which he had recently seen a case in the Balkans, where he was at the time as Chief Physician to the Anglo-Serbian Military Hospital, Sotoriotissa. From all the Ceylon cases, one of which appeared to have started while the patient was employed on a plantation in the Federated Malay States, he grew the same fungus which, he said, there could be no doubt was the cause of the disease. Cultures of a Ceylon strain were given to Prof. E. Pinoy of the Pasteur Institute, Paris, and Pinoy's description of it is cited in Castellani's paper.

The fungus was of somewhat difficult classification but Pinoy proposed to include it, at least temporarily, in the genus *Acladium* (misspelt *Accladium* in the transcription) and call it *A. castellanii*. On Sabouraud's agar and carrot the colonies were whitish and amber-yellow and formed many small roundish masses which later often coalesced, covered by spiculated formations consisting of erect, straight filaments parallel to each other or at times interlacing. These filaments were approximately 2μ broad and carried laterally pseudoconidia of variable shape, cylindriform, piriform, or spherical, attenuated at their points of insertion. Most of these pseudoconidia were 4 by 3μ. This type of fructification recalled the type *Acladium* described by Bodin in some species of *Trichophyton*. The pseudoconidia become detached and then develop by sprouting, and mycelial filaments are formed. Certain filaments produce spherical chlamydospores arranged in small strings as in some species of *Fusarium*, very frequently terminal, and variable in size, $8–10\mu$.

The figures show that the "pseudoconidia" are also sometimes produced terminally at a hyphal tip or on lateral branches of variable length, though this is not mentioned in the description; they are shown as single and not in clusters.

The disease is described by Castellani as a clinically characteristic ulceration which was easily reproduced in two coolies (who volunteered) by injecting cultures of the isolated fungus. The ulcers may occur practically all over the body, though the face, scalp and palmar surfaces often escape. They are generally sharply defined, roundish or oval, with red granulating fundus. Gummatous nodules and furuncle-like lesions may also be observed and some of the superficial lymphatic glands are often enlarged. The lesions are not, as

a rule, very painful and there is little or no itching. The general condition of the patient is not seriously affected for a long time, but he often complains of weakness and general discomfort, and is unfit to attend to his work. The course of the disease is long and there is not much tendency to spontaneous cure. Most of the cases were originally diagnosed as syphilis but in only one was there a history pointing to that disease; the Wassermann reaction was negative in two cases tested, spirochaetes were not found, and salvarsan and mercury were not beneficial, while a fairly rapid cure was obtained with potassium iodide in full doses, especially if given in a salt-free diet. Isolations from lesions not yet ulcerated yielded only the fungus.

In a later paper, published in 1917, Castellani(2) termed the disease "accladiosis" (corrected to "acladiosis" in the Milroy Lectures(3) in 1920) and gave further details. He stated that cases had been very recently observed in the Balkanic zone by himself, Laurie and others. There is little or no pain but occasionally slight pruritis. In a Macedonian case with abundant purulent secretion from the ulcers there was fever reaching on some days 102 or 103° F. An incomplete histo-pathological investigation showed that the lesions are very similar to those seen in sporotrichosis and may be of three types: epithelioid or tuberculoid, lympho-connective (syphiloid), and poly-morpho-nuclear (ecthymatous). In culture on carrot and potato the fungal colonies are white, on glucose agar often amber colour. Very old cultures may show a certain amount of pigmentation. A positive diagnosis, e.g. from sporotrichosis and other affections of hyphomycetic origin, can be made with certainty only by cultural methods. In the figures of a young culture on carrot and an old one on potato accompanying this paper, coremial formation can be seen, and in the last-named the growth appears to be dark or black.

In 1920 Mendelson(4) described very briefly another case of this disease which he observed that year at the Central Hospital, Bangkok, Siam, in a Chinese coolie working in a local mill. The ulcers appeared first on the knee and subsequently spread to the leg near by. There was slight itching but little discomfort, no pain, and no glandular enlargement. The ulcers were sharply depressed, with clearly defined borders and red granulating tissue at the base. A fungus, stated to have all the morphological and cultural characters of *Acladium castellanii*, was isolated from the ulcers and gave a creamy abundant growth on carrot and potato, which turned darker in a few days.

The only other record that the writer has traced is one by Da Fonseca & Leão(5) in Brazil in 1927. The fungus was isolated from ulcers on the jaw and presternal region, and formed smooth, shining, yellowish colonies on Sabouraud's medium. The hyphae were slender and bore ovoid or elongated aleuriospores laterally or at the tips. No chlamydospores were seen.

In 1923 Dr R. Craik of Ealing published an account(6) of his cultural studies of *Acladium castellanii*, which he grew from a culture sent to him by Castellani. On glucose or wort agar it formed a whitish pasty layer, turning brown in a month. These "smooth" cultures were formed at winter room

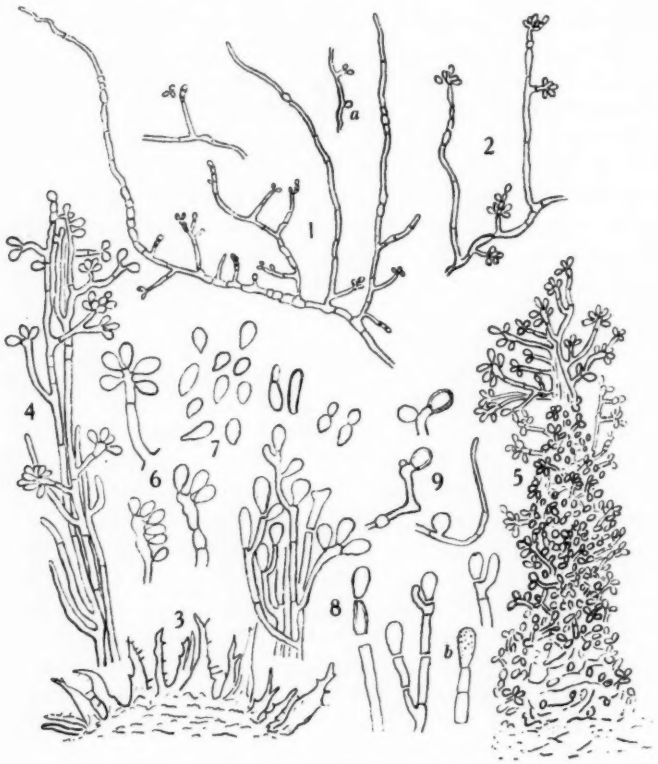
temperature (10–15° C.) and did not develop structurally differentiated conidiophores or conidia. The hyphae were 2–3 μ broad and consisted of long cells except in parts where rather close septa divided the hypha into oidial elements ("pseudoconidia"). There were also short moniliform lengths of hyphae, often at the end of a branchlet. Many spherical or ovoid, unicellular chlamydospores, of a much greater diameter than the hyphae, developed intercalarly or terminally, sometimes on short lateral offshoots. Less frequently, elements comparable in size to the chlamydospores, but elongated and with one or two septa, formed, usually on short lateral branches. The "rough" cultures developed on the same media at 30° C. They became covered in a week with tiny spicules formed of fasciculate coremia of hyphae, branching near the tip to bear numerous solitary, terminal, colourless, piriform conidia, 2 by 3 μ in diameter and with a facet of articulation when detached. In the "rough" cultures chlamydospores were rare, while in the "smooth" ones conidia were not seen.

Craik considered the fungus to be more closely allied to *Microsporon* (though it could not be placed in that genus) than to *Acladium*, and renamed it *Pseudomicrosporon castellanii*.

The writer received cultures of the fungus from Craik and Castellani and grew it at the Imperial Mycological Institute, Kew, on various natural substrata (carrot, potato, etc.) and on Dox's agar and other laboratory media. The best growth was obtained on carrot and potato. On carrot the cultures were white or gradually changed to yellow; on potato they were white at first but a change to deep brown or black occurred at a later stage (Pl. I), even when the culture was derived from a strain that continued to give a white or yellowish growth on carrot. In the early stages of development there was some indication of the *Acladium* type of sporing (Text-fig. 1), but in vigorous cultures this was soon lost and clusters of conidia developed at the ends of the lateral branches (Text-fig. 2). Tapering coremia formed at a later stage in strong cultures, especially on carrot (Pl. I; Text-figs. 3–5), and on these, conidia were produced very copiously; they developed at and near the ends of the terminal branching hyphae and along the sides of the coremia on the ends of lateral branches from the main coremial strands. The coremia are composed of broader and straighter hyphae than the slender flexuous ones of the creeping mycelium (Text-fig. 4). Anastomoses were not seen.

The conidia (Text-figs. 6, 7) are mainly piriform, sometimes oval, colourless, and measure 4·5–7 by 3–4 μ. They are inserted directly on the hypha or on small tooth-like processes which occasionally may be long enough to constitute a kind of stalk. The facet of articulation mentioned by Craik is not usually visible in the aerial conidia formed in strong cultures but can be readily identified in the conidium-like bodies formed on submerged hyphae (Text-fig. 8). In vigorous cultures the vast majority of the conidia are formed in clusters at and close to the ends of the sporing hyphae; laterally inserted conidia along the length of the hyphae, as in *Acladium*, are rarely seen. No

clear evidence was obtained that the conidia are ever formed in chains, but the heads may contain so many spores that the occurrence of chains is difficult to disprove. With a fungus believed to be strongly pathogenic to man, the



Acladium castellanii Pinoy. From cultures on vegetable organic media.

Text-fig. 1. Young mycelium with spores. At *a*, *Acladium* type. × about 166.
Text-fig. 2. Slightly more advanced stage. × 233.
Text-fig. 3. Spicules formed on surface of medium. × about 10.
Text-fig. 4. Coremium formation. × 320.
Text-fig. 5. Upper part of a sporiferous spicule. × 233.
Text-fig. 6. Three spore-bearing hyphal tips showing insertion of spores. × 566.
Text-fig. 7. Spores. Three towards the right possibly germinating. × 566.
Text-fig. 8. Submerged fragmenting mycelium. At *b*, a roughened spore-like segment. × 566.
Text-fig. 9. Chlamydospores. × 320.

following of developmental stages by continuous observation of living material in active growth is hampered; hanging-drop cultures did not spore sufficiently freely to give conclusive evidence regarding chain formation, but as budding conidia were occasionally seen there is a possibility that the apparent chains

of two or three cells sometimes seen near the ends of the sporing hyphae were formed by acropetal budding.

Chlamydospores were not seen in the aerial sporing cultures but were found in the creeping, and especially in the submerged growth (Text-fig. 9). They were unicellular, rather thick-walled bodies formed terminally, intercalarly or laterally, and more or less spherical, except for certain club-shaped terminal ones which were considerably longer than broad and occasionally had roughened walls (Text-fig. 8*b*). The submerged mycelium tends to disarticulate into hyphal segments; and spore-like bodies, which somewhat resembled the aerial conidia but had a broad facet of disarticulation, were borne on hyphae at and below the surface of the medium (Text-fig. 8).

The aerial conidia seem to belong to the type termed radula spores by E. W. Mason in his studies of conidial forms of the Hypocreales (7). They are not, I think, *conidia vera* but thallospores; indeed, if the spore-like bodies of the submerged mycelium (Text-fig. 8) are the same organs as the aerial conidia, save for modifications due to differences of habit, this conclusion can scarcely be resisted. In the absence of other known spore forms identification even to the genus is difficult, but a comparison with other human pathogens and the lesions they cause indicates the affinities of the fungus.

The fungus closely resembles the descriptions of the human and animal pathogens of the form genus *Sporotrichum* (*Rhinocladium* of some authors), and there is little in the clinical characters of acladiosis to distinguish this disease from sporotrichosis. Systematically, the causal organism must be referred to the genus *Sporotrichum* to which, if the question of colour be excluded, most of the truly ulcerative hyphomycetes (as distinct from the yeast-like fungi) that have been described belong. Colour is an unreliable criterion in this group for, as mentioned above, white and black strains may be grown from a single isolation and the same is true for the commonest cause of human and equine sporotrichosis, *Sporotrichum schencki*. The clinical similarity of acladiosis to sporotrichosis is apparent from the descriptions, especially the formation of gummata, marked absence of pain, and slight general upset in the chronic forms. It is true that Castellani, in a letter to the writer, claimed that there is a difference in the appearance of the ulcers, but this cannot be very marked as he had earlier stated that cultural methods were necessary to distinguish the two diseases. Histo-pathologically the resemblance is so close that Castellani recognizes the same three types of lesion in acladiosis that were described for sporotrichosis in the third edition of the *Manual of Tropical Diseases* by himself and Chalmers in 1919.

In comparing the fungus described above with the known human pathogens of the genus, the similarity with the description of *Sporotrichum schencki* Hektoen & Perkins given by D. J. Davis (8), who has made one of the most complete cultural studies of this species, is striking. This fungus was first isolated by Schenck (9) in the United States in 1898 and was identified by Erwin F. Smith as one of the Sporotricha. In 1900 Hektoen & Perkins (10)

named it *Sporothrix* (*sic*) *schencki*, the name being corrected to *Sporotrichum schencki* by Matruchot in 1910. Davis compared cultures derived from those of Hektoen isolated in 1899 with cultures of the subsequently described *S. beurmanni* Matruchot & Ramond 1905, received from Gougerot and Sabouraud in Paris, and with others from American sources, and decided that all were identical. He concluded that *S. indicum* Castellani and *S. jeanselmei* Brumpt & Langeron were also probably identical with *S. schencki*. Vuillemin (11) also thinks that *Rhinocladium* (the generic name preferred by him) *schencki* and *R. beurmanni* are probably identical, while Dodge (12) regards *Sporotrichum beurmanni* as merely a variety of *S. schencki*.

Davis describes the tendency in all the strains of *S. schencki* that he studied to the formation of hair-like processes or finely pointed spines on the surface of the medium, and the colour changes from pure white to brown or black. The formation of coremia and change from creamy to tan and black is also mentioned in *S. beurmanni* by Benham & Kesten (13). The spore characters as described by all these authors and as figured admirably by Moore in Fig. 122 of Dodge's *Medical Mycology* show little difference from those of Castellani's fungus.

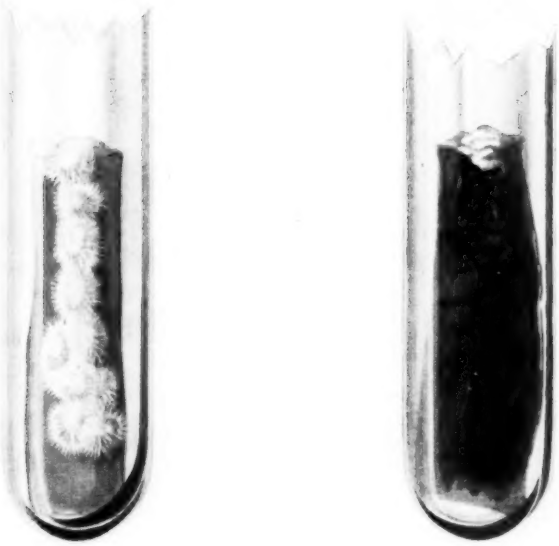
It is probably unsafe without a comparative cultural study of a range of the human and animal pathogens that have been referred to the genus *Sporotrichum* to attempt a closer identification of the species here described. The tendency to saltation stressed by Davis and others who have grown these fungi in culture, and the manner in which the saltants of one so-called species assume the characters of another, indicate that the definition of the limits of species within the group presents unusual difficulties. It seems probable that the limits must be drawn wide, and that *S. schencki* will be found to include a large proportion of the "new species" of this genus since recorded as human and animal pathogens. If *S. councilmani* is to be so included, and Dodge regards it as only a variety of *S. schencki*, then it is likely that "*Acladium castellanii*" is no more than a form of this world-wide fungus, for a diagnosis of *S. schencki* that could include *S. councilmani* would also readily include *Acladium castellanii*.

In any case there is no doubt that acladiosis should be deleted as a distinct disease and merged with the sporotrichoses.

SUMMARY

Previous records and descriptions of the fungus known as *Acladium castellanii*, a human pathogen in the south-east of Asia and Europe and in Brazil, are cited and an account is given of its morphology when studied in culture on organic media.

It is concluded that it is closely related to, and perhaps merely a strain of, the widely distributed *Sporotrichum schencki*, the commonest cause of human and equine sporotrichosis, and that the term acladiosis as denominating a distinct disease should be deleted.



Acladium castellanii Pinoy. Left on carrot. Right on potato.

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EXPLANATION OF PLATE XIV

Acladium castellanii Pinoy. Left on carrot; right on potato.

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AN IMPROVED METHOD OF INOCULATING PLANTS WITH VIRUS FOR THE STUDY OF LOCAL LESIONS

By W. D. MacCLEMENT

From the Molteno Institute and Potato Virus Research Station, University of Cambridge

(With 3 Figures in the Text)

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I. Introduction

The estimation of the amount of virus in a given suspension by counting the local lesions would be a useful technique if the method was sufficiently accurate to allow of a direct interpretation of results.

The method was introduced by Holmes in 1929, who found that local lesions were produced on a plant if a suitable leaf was rubbed with a piece of cloth, saturated with the infective fluid, just sufficiently hard to break the hairs of the leaf. The number of these lesions produced was related to the virus concentration of the fluid.

In 1931 Samuel simplified the inoculation method by substituting a spatula with a ground-glass face for the cloth used by Holmes. In 1933, with Bald, he further improved the accuracy of the method by inoculating half the leaf with the fluid and the other half with the control and randomizing the inoculations. Youden & Beale in 1934 and Bald in 1935 analysed the method statistically and were able by careful arrangement of the experiments, involving many repetitions, to produce reliable figures.

The method still remained, however, far from ideal and with the object of making it more accurate as well as less tedious a careful study of the technique was undertaken.

II. Experimental

The virus used in this study was a "green" strain of tobacco mosaic (Johnson's Tobacco Virus I). This strain had been carefully isolated from Jensen's Yellow Tobacco Mosaic kindly supplied by Dr Kenneth Smith.

The test plant used was *Nicotiana glutinosa*. This species produces only local lesions when inoculated with the tobacco mosaic virus and so is very suitable for the application of the local lesion technique. The plants were grown in 5 in. pots and when large enough the flower buds and lower leaves were removed, leaving five or six fully expanded leaves of even size.

Analysis of the results of a number of previous inoculations using the old method had shown that the irregularities were due to a combination of several variables. An attempt was made to separate and estimate these.

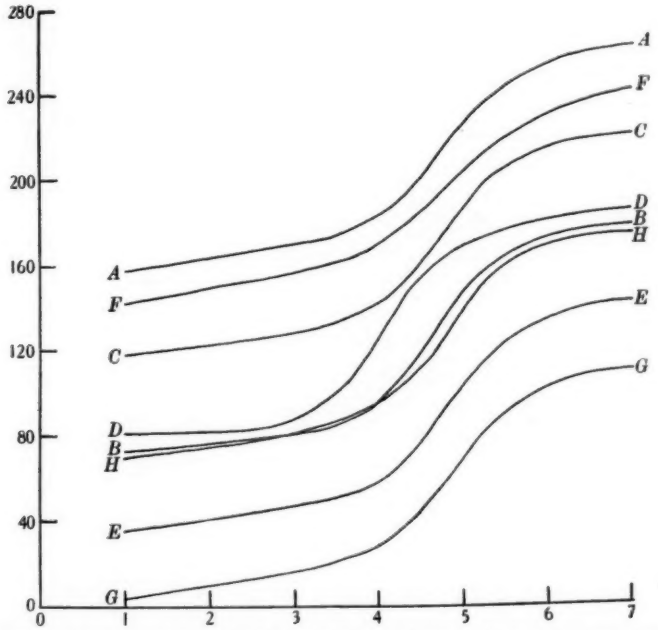

Fig. 1. Curves showing response of eight *N. glutinosa* plants *A–H* to a standard inoculum of tobacco mosaic. Ordinates = leaf no. from top of plant downwards. Abscissae = lesions per leaf.

III. The variables

(1) *The plant*

The variation in response shown by *N. glutinosa* plants to a standard inoculum is the largest of the variables and can be as much as 100 per cent above or below the mean. In Fig. 1 are shown the results of inoculating seven leaves on each of eight plants. These plants were grown during the summer under the ordinary glasshouse conditions and were as uniform in appearance as plants of this species can be. Nevertheless the variation in response to the virus inoculum was very wide. The lowest number of lesions was 2 on leaf number 1 of plant *G* while the highest was 264 on leaf number 7 of plant *A*.

The mean was found to be 138 lesions and the variation was about 100 per cent of this.

(2) *The leaf*

The type of response to inoculation shown by individual leaves on a plant is clearly indicated by the nearly parallel lines of Fig. 1. This response is typical for plants grown under similar conditions. It seems clear that the type of response of all these plants is similar and that it is the level of response that varies. Further evidence of this is given in Fig. 2, where the sums of

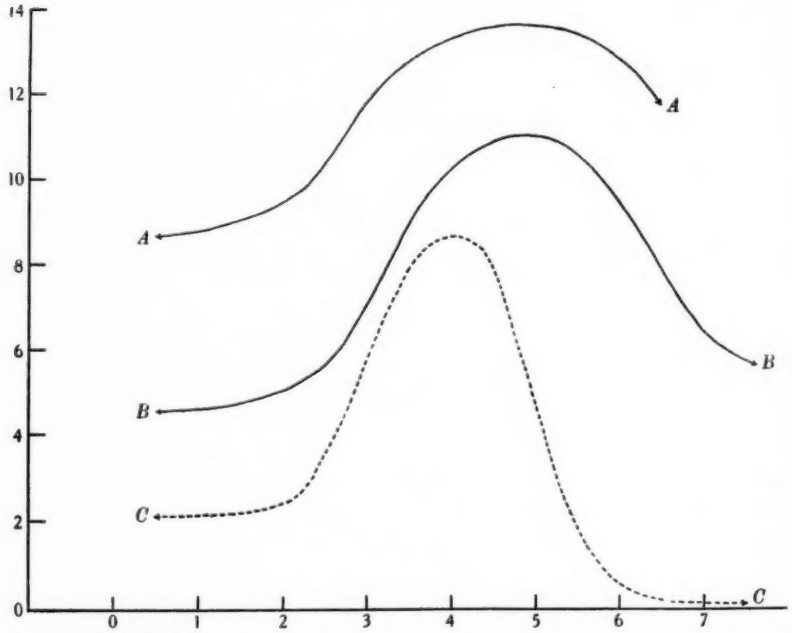

Fig. 2. Curves showing total response of *N. glutinosa* plants: $A = 100$ in summer, $B + C = 100$ in winter to $A + B$ = tobacco mosaic virus, C = "X" potato virus. Ordinates = leaf no. from top of plant downwards. Abscissae = thousands of lesions per leaf.

the counts for corresponding leaves are represented. The difference in response to inoculation in summer and winter respectively is again one of level rather than of type. It is worth recording that the curve for potato virus "X" shows a difference in level as well as in type. It is not possible to obtain an accurate estimate of the virus concentration of different samples of infective fluids by counting the local lesions produced on the leaves of plants which have been grown under different conditions and chosen at random. The level of inoculation response of any one leaf in relation to the next leaf, either above or below, will not be constant.

(3) *The leaf hairs*

The third variable to be studied was the number of hairs available for inoculation on a leaf and the percentage of these broken during the inoculation process. On the upper surface of an average fully expanded leaf of *N. glutinosa* with an area of 15 square inches there are about 35,000 hairs. From drawings of marked areas of the leaf surface before inoculation and after the formation of local lesions, it appears that any hair can be a point of entry for the virus if broken under the right conditions. Using a glass spatula the number of hairs broken varies from 40 to 73 per cent of the total available. Wiping each side of the midrib once with the spatula gave very low counts, usually below 30 per cent, the average being fairly constant at about 27 per cent. Rubbing with a wad of cotton wool gave an average of 60 per cent of broken hairs, but there was much irregularity and some damage to the leaf surface. Rubbing with cheesecloth was better; this gave an average of just over 75 per cent of the hairs broken and there was little damage to the leaf. The best results were obtained with the finger, which could be rubbed gently over the leaf without damage. This method gave an average of 83 per cent of the available hairs broken, but had to be abandoned because it was tedious and because of the difficulty of sterilization.

(4) *The inoculum*

The amount of liquid used during each inoculation varied with the method. To produce a given number of lesions the cotton wool used the largest amount; the glass spatula used the least fluid and was the most consistent in the quantity used.

(5) *Amount of rubbing*

The fifth variable was linked to the fourth. For any of the methods there appeared to be an optimum amount of rubbing for a given volume of liquid. The method which needed the least rubbing to break the maximum number of hairs involved, gave the most consistent results with a given volume of liquid. The best method, therefore, was found to be the use of the glass spatula, rubbed with short strokes in one direction only, parallel to and between the side veins of the mid-rib. From this it was concluded that the more mechanical the method, the better the results would be.

IV. Summary of results

The summary of the investigation is as follows: (*a*) The level of inoculation response by *N. glutinosa* is characteristic of one plant alone and may vary greatly from that of other plants of the same kind. (*b*) The type of inoculation response of a plant, i.e. the relation of one of its leaves to the others on the same plant, is typical of the conditions under which that plant is grown. (*c*) For any one set of conditions the type of inoculation response of the individuals of a group of plants is the same. (*d*) The very large number of

hairs available for virus entry, compared with the number of local lesions produced, tends to increase the irregularity of results unless a constant percentage of hairs is broken. (*e*) The variable due to irregularities in the amount of liquid inoculum used and the length of movement of this liquid during inoculation is decreased as the method is made more mechanical.

There seemed, thus, to be scope for improvement in the technique, especially if it could be simplified. All the results outlined above were studied from the point of view of finding the ideal method. Various mechanical appliances were tried until one was evolved which satisfied most of the requirements.

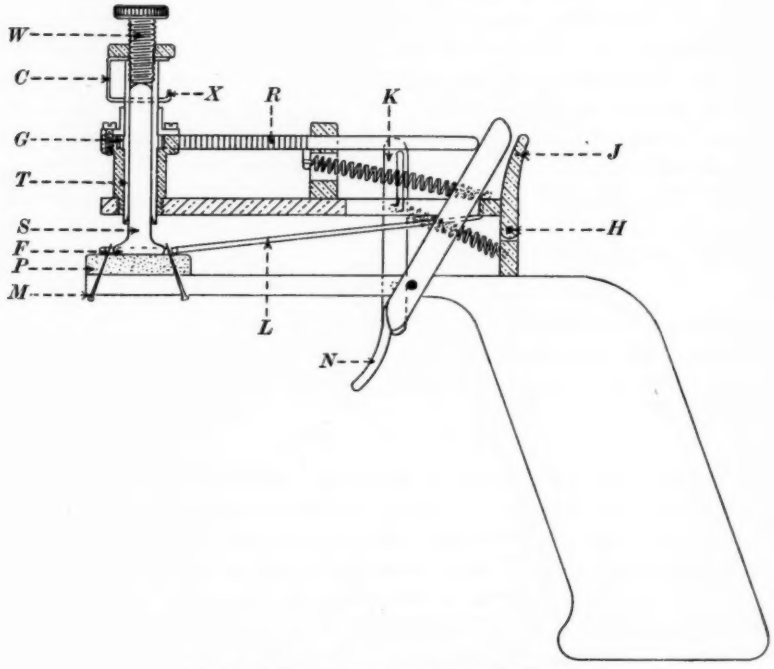

Fig. 3. Sectional view of inoculation apparatus.

V. Description of the apparatus

The apparatus, as finally designed, is completely mechanical and it eliminates so far as possible the personal element. It consists of a small pistol-like mechanism which rotates a small ground glass spatula against the surface of the leaf. The apparatus is illustrated in Fig. 3. The glass spatula *S* is held in the tube *T* by the clamp *C*. The tube is rotated by the gear *G* and ratchet *R* when the trigger *N* is pulled. The apparatus opens on hinge *H*, when the catch *K* is moved back, to allow the leaf to be inserted between the face

of the spatula *F* and the sponge-rubber pad *P*. The apparatus is closed on the leaf by pressing *J*. In doing this the leaf is pressed against the pad by the spring *L*. The four pins *M* pierce the leaf and hold it in position. If their points are bent slightly inwards, they help to stretch the leaf and keep it flat. The punctures serve subsequently to mark the place of inoculation. *J* is pressed until the catch *K* slips into position and holds everything ready for inoculation. The maximum number of local lesions is produced when the pressure of the spatula is adjusted, by means of the screw *W*, to break all the leaf hairs close to their base without marking the epidermis. A number of spatulae can be used without readjusting *W* if they are ground till their overall lengths are the same. They are released by pressing the clamp at *X* when the apparatus is open. They are easily sterilized by dropping them into boiling water. With this apparatus a very small amount of inoculum is necessary, for a spatula with a circular head half an inch in diameter 0·001 c.c. is the best quantity to use. The optimum number of rotations of the spatula under these conditions is three.

The small drop of inoculum is placed on the leaf by means of a simple type of syringe which consists of a 0·02 c.c. glass pipette of the kind used in opsonic work and graduated in hundreds. The plunger, made of a brass spring wire, can move a column of liquid in the pipette with great accuracy. Half an inch of air space should be kept between the end of the wire and the upper end of the column of liquid. Readings are taken from the meniscus and not the movement of the wire. The tip of the pipette is ground to a fine point and waxed; this allows the small drop of inoculum to be transferred easily to the correct place on the leaf. A lump of sealing wax on the upper end of the wire provides a convenient handle. The method of procedure is as follows: the inoculum is drawn up into the pipette to a predetermined mark, the tip is then wiped dry and the plunger moved down till the desired amount of fluid is ejected. The surface of the leaf is touched with the tip of the pipette midway between two of the side veins, where there is at least half an inch of smooth surface. Next the leaf is inserted in the apparatus so that the drop of inoculum is directly under the spatula. The apparatus is then closed and the trigger pulled to its full extent and released three times. The instrument is then opened and removed from the leaf. The whole operation takes about 5 sec., and in that time a measured volume of inoculum can be rubbed a fixed number of times over a known area of leaf under constant pressure. Practically 100 per cent of the hairs involved are broken without injuring the leaf epidermis.

This method has the disadvantage of requiring rather a complex apparatus, but this is offset by the fact that it eliminates much of the personal factor which was largely the cause of the irregularities in the previous methods. An important advantage of the method is the fact that it allows several samples of material to be compared on one leaf. The average number of inoculations is 12 but as many as 25 separate inoculations can be made on a large leaf. This means that a whole experiment can be carried out on one plant with as many repeat inoculations as there are leaves on the plant.

The writer's experience has been that by means of this apparatus it is possible to carry out in 3 weeks experiments which would have taken 3 months using the old methods, for the variation in results is less than 10–20 per cent. Briefly then the advantages of this method of inoculation seem to be the production of results which need little mathematical interpretation, and the saving of time, patience and plants.

VI. Summary

An account is given of an investigation of the causes of error in estimating virus concentrations by the local lesion method. The irregularities were found to be due to:

(1) Variations in the response of (*a*) whole plants; (*b*) individual leaves.

(2) Variations in the volume of liquid used.

(3) Variations in the probability of the production of local lesions.

(4) Variations in the percentage of leaf hairs broken during the inoculation process.

An apparatus is described, the use of which reduces these irregularities to a minimum.

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